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(54) Title: INDUCTION OF CTL RESPONSES TO FOREIGN ANTIGENS EXPRESSED IN MYCOBACTERIA

(57) Abstract

A method of inducing a CTL response in an animal which comprises administering to the animal mycobacteria transformed with at least one DNA sequence which encodes a protein or peptide or fragment or derivative thereof which includes an epitope which is recognized by cytotoxic T lymphocytes. The mycobacteria are administered in an amount effective to induce a CTL response in an animal. In one embodiment, the transforming DNA sequence may encode an HIV protein or fragment or derivative thereof.

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INDUCTION OF CTL RESPONSES TO FOREIGN ANTIGENS EXPRESSED IN MYCOBACTERIA

This invention relates to the induction of a T-cell response, in particular a cytotoxic T lymphocyte response. More particularly, this invention relates to the induction of CTL responses to proteins or polypeptides expressed by recombinant mycobacteria.

Cell-mediated immunity (or CMI) of infections is thought to be a major line of defense against certain infections, such as viral infections and certain bacterial infections. For example, CMI may be significant in the development of an effective vaccine against human immunodeficiency virus (HIV), or AIDS virus, because HIV vaccines and/or therapies based on the generation of passive transfer of HIV-specific antibody in the absence of cell-mediated immunity have not yielded consistent protection in primates challenged with the HIV virus. Thus, interest has turned to the induction of cell-mediated responses to various infections, such as for example, HIV infection, and to the identification of proteins or polypeptides that a stimulate a cytotoxic T lymphocyte response, and to methods of administering such proteins or polypeptides.

In accordance with an aspect of the present invention, there is provided a method of inducing a CTL response in an animal comprising administering to the animal mycobacteria transformed with at least one DNA sequence which encodes a protein or peptide or fragment or derivative thereof which includes an epitope which

is recognized by cytotoxic T lymphocytes. The mycobacteria are administered in an amount effective to induce a CTL response in an animal.

Proteins or polypeptides for which the at least one DNA sequence may encode, include, but are not limited to, Mycobacterium leprae antigens; Mycobacterium tuberculosis antigens; Rickettsia antigens; Chlamydia antigens; Coxiella antigens; malaria sporozoite and merozoite proteins, such as the circumsporozoite protein from Plasmodium berghei sporozoites; Clostridium antigens; Leishmania antigens; Salmonella antigens; Mycobacterium africanum antigens; Mycobacterium intracellulare antigens; Mycobacterium avium antigens; E.coli antigens; Borrelia antigens; Listeria antigens; Franciscella antigens; Yersinia antigens; Treponema antigens; Schistostoma antigens; Filaria antigens; Pneumococcus antigens; Staphylococcus antigens; Herpes virus antigens; influenza and parainfluenza virus antigens; measles virus antigens; mumps virus antigens; hepatitis virus antigens; Shigella antigens; Bordatella antigens; Hemophilus antigens; Streptococcus antigens; polio virus antigens; Rift Valley Fever virus antigens; dengue virus antigens; Human Immunodeficiency Virus (HIV) antigens; and respiratory syncytial virus (RSV) antigens.

In one embodiment, the at least one DNA sequence encodes at least one protein or polypeptide or fragment or derivative thereof which includes an eptiope which is recognized by cytotoxic T lymphocytes induced by an HIV protein or fragment or derivative thereof. The at least one DNA sequence may encode an HIV protein or fragment or derivative thereof. HIV proteins or polypeptides which may be encoded by the at least one DNA sequence includes but are not limited to, HIV-I-gp 120; HIV-I-gp 41; HIV-I-gp 160; HIV-I-pol; HIV-I-nef; HIV-I-tat; HIV-I-rev; HIV-I-vif; HIV-I-vpr; HIV-I-vpu; HIV-I-gag; HIV-2-gp 120; HIV-2-gp 160; HIV-2-gp 41; HIV-2-gag; HIV-2-pol; HIV-2-nef;

HIV-2-tat; HIV-2-rev; HIV-2-vif; HIV-2-vpr; HIV-2-vpu; and HIV-2-vpx.

Mycobacteria which may be transformed with the at least one DNA sequence, which encodes a protein or polypeptide or fragment or derivative thereof which includes an epitope which is recognized by cytotoxic T lymphocytes, include, but are not limited to, Mycobacterium bovis-BCG, M.smegmatis, M.avium, M.phlei, M.fortiutum, M.lufu, M.paratuberculosis, M.habana, M.scrofalaceum, and M.intracellulare. In a preferred embodiment, the mycobacterium is M.bovis-BCG or a mutant thereof.

The at least one DNA sequence may be contained within an expression vector, which is transformed into a mycobacterium, whereby the mycobacterium expresses the protein or polypeptide or fragment or derivative thereof which includes an epitope which is recognized by cytotoxic T lymphocytes.

The expression vector may be, for example, a temperate shuttle phasmid or a bacterial-mycobacterial shuttle plasmid. Each of these vectors may be used to introduce the at least one DNA sequence encoding a protein or polypeptide or fragment or derivative which includes an epitope which is recognized by cytotoxic T lymphocytes, stably into mycobacteria, in which the at least one DNA sequence may be expressed. When a shuttle phasmid, which replicates as a plasmid in bacteria and a phage in mycobactreia, is employed, integration of the phasmid, which includes the at least one DNA sequence encoding a protein or polypeptide, or fragment or derivative thereof, which includes an epitope which is recognized by cytotoxic T lymphocytes, into the mycobacterial chromosome occurs through site-specific integration. The at least one DNA sequence which encodes a protein or polypeptide or fragment or derivative thereof, which includes an epitope which is recognized by cytotoxic T lymphocytes, is replicated as part of the chromosomal DNA. When a bacterial-mycobacterial shuttle plasmid is employed, the at least one DNA sequence which encodes a protein or polypeptide or

fragment or derivative thereof, which includes an epitope which is recognized by cytotoxic T lymphocytes, is stably maintained extrachromosomally in a plasmid. Expression of the at least one DNA sequence occurs extrachromosomally (e.g., episomally). For example, the at least one DNA sequence is cloned into a shuttle plasmid and the plasmid is introduced into a mycobacterium such as those hereinabove described, wherein the plasmid replicates episomally. Examples of such shuttle phasmids and bacterial-mycobacterial shuttle plasmids are further described in Application Serial No. 361,944, filed June 5, 1989, which is hereby incorporated by reference.

In one embodiment the mycobacteria are transformed with an expression vector which comprises at least one DNA sequence encoding a protein or polypeptide which includes an epitope which is recognized by cytotoxic T lymphocytes, and a promoter selected from the class consisting of mycobacterial promoters and mycobacteriophage promoters for controlling expression of the DNA encoding the heterologous protein or polypeptide, or fragment or derivative thereof, which includes an epitope which is recognized by cytotoxic T lymphocytes.

Mycobacterial and mycobacteriophage promoters which may be employed include, but are not limited to, mycobacterial promoters such as the BCG HSP60 and HSP70 promoters; the mycobactin promoter from M. tuberculosis and BCG; the mycobacterial 14 kda and 12 kda antigen promoters; the mycobacterial α-antigen promoter from M.tuberculosis or BCG; the MBP-70 promoter, the mycobacterial 45 kda antigen promoter from M.tuberculosis or BCG; the superoxide dismutase promoter; the mycobacterial asd promoter, and mycobacteriophage promoters such as the Bxb1, L1, L5, and TM4 promoters. In one embodiment, the promoter is a mycobacterial heat shock protein promoter such as HSP60 or HSP70.

The promoter sequence may, in one embodiment, be part of an expression cassette which also includes a portion of the gene normally under the control of the promoter. For example, when a

mycobacterial HSP60 or HSP70 promoter is employed, the expression cassette may, within the scope of the present invention, include, in addition to the promoter, a portion of the gene for the HSP60 or HSP70 protein. When the expression cassette and the DNA encoding the protein or polypeptide, or fragment or derivative thereof, which includes an epitope which is recognized by cytotoxic T lymphocytes are expressed, the protein expressed by the cassette and the DNA encoding the protein or polypeptide is a fusion protein of a fragment of a mycobacterial protein (eg., the HSP60 or HSP70 protein), and the protein or polypeptide or fragment or deriviative thereof which includes an epitope which is recognized by cytotoxic T lymphocytes.

In a preferred embodiment, the transcription initiation site, the ribosomal binding site, and the start codon, which provides for the initiation of the translation of mRNA, are each of mycobacterial origin. The stop codon, which stops translation of mRNA, thereby terminating synthesis of the protein or polypeptide or fragment or derivative thereof which includes an epitope which is recognized by cytotoxic T lymphocytes, and the transcription termination site, may be of mycobacterial origin, or of other bacterial origin, or such stop codon and transcription termination site may be those of the DNA encoding the protein or polypeptide which includes an epitope which is recognized by cytotoxic T lymphocytes.

Preferably, the mycobacterial promoter is a BCG promoter, and the mycobacterium is BCG.

In one embodiment, the expression vector may further include DNA which encodes for proteins or polypeptides such as, but not limited to, antigens, anti-tumor agents, enzymes, lymphokines, pharmacologic agents, immunopotentiators, reporter molecules of interest in a diagnostic context, and selectable markers.

Selectable markers which may be encoded include, but are not limited to, the β-galactosidase marker, the kanamycin resistance

marker, the chloroamphenicol resistance marker, the neomycin resistance marker, and the hygromycin resistance marker.

In accordance with one embodiment, the vector further includes a mycobacterial origin of replication.

In accordance with another embodiment, the vector may be a plasmid. The plasmid may be a non-shuttle plasmid, or may be a shuttle plasmid which further includes a bacterial origin of replication such as an E.coli origin of replication, a Bacillus origin of replication, a Staphylococcus origin of replication, a Streptomyces origin of replication, or a pneumococcal origin of replication. In one embodiment, the shuttle plasmid includes an E. coli origin of replication.

In accordance with yet another embodiment, the vector may further include a multiple cloning site, and the DNA encoding for the protein or polypeptide, or fragment or derivative thereof, which includes an epitope which is recognized by cytotoxic T lymphocytes is inserted in the multiple cloning site.

In addition to the DNA encoding a heterlogous protein or polypeptide, and the mycobacterial promoter for controlling expression of the DNA encoding the protein or polypeptide which includes an epitope which is recognized by cytotoxic T lymphocytes, the expression vector may, in one embodiment, further include a DNA sequence encoding bacteriophage integration into a mycobacterium chromosome. Bacteriophages from which the DNA sequence encoding bacteriophage integration into a mycobacterium chromosome may be derived include, but are not limited to, mycobacteriophages such as but not limited to, the L5, L1, Bxb1, and TM4 mycobacteriophages; the lambda phage of E. coli; the toxin phages of Corynebacteria; phages of Actinomycetes and Norcardia; the ϕC31 phage of Streptomyces; and the P22 phage of Salmonella. Preferably, the DNA sequence encodes mycobacteriophage integration into a mycobacterium chromosome. The DNA sequence which encodes bacteriophage integration into a mycobacterium chromosome may include DNA which encodes integrase,

which is a protein that provides for integration of the vector into the mycobacterial chromosome. Preferably, the DNA sequence encoding mycobacterial phage integration also includes DNA which encodes an attP site.

The DNA encoding the attP site and the integrase provides for an integration event which is referred to as site-specific integration. DNA containing the attP site and the integrase gene is capable of integrating into a corresponding attB site of a mycobacterium chromosome.

It is to be understood that the exact DNA sequence encoding the attP site may vary among different phages, and that the exact DNA sequence encoding the attB site may vary among different mycobacteria.

Examples of expression vectors which include mycobacterial promoters and mycobacteriophage promoters, for controlling the at least one DNA sequence encoding a protein or polypeptide, or fragment or derivative thereof, which includes an epitope which is recognized by cytotoxic T lymphocytes are further described in application Serial No. 642,017, filed January 16, 1991, which is a continuation of Application Serial No. 552,828, filed July 16, 1990, now abandoned. The contents of Application Serial No. 642,017 are hereby incorporated by reference.

In another embodiment, the mycobacteria are transformed with DNA which comprises a first DNA sequence which is a phage DNA portion encoding bacteriophage integration into a mycobacterium chromosome, and DNA including the at least one DNA sequence encoding a protein or polypeptide, or fragment or derivative thereof, which includes an epitope which is recognized by cytotoxic T lymphocytes.

The term "phage DNA portion", as used herein, means that the DNA sequence is derived from a phage and lacks the DNA which is required for phage replication.

Bacteriophages from which the phage DNA portion may be derived include, but are not limited to, mycobacteriophages, such

as but not limited to the those hereinabove described. Preferably, the phage DNA portion encodes mycobacteriophage integration into a mycobacterium chromosome.

In a preferred embodiment, the first DNA sequence includes DNA encoding integrase, which is a protein that provides for integration of the DNA into the mycobacterial chromosome. Most preferably, the first DNA sequence also includes DNA which encodes an AttP site.

The DNA sequence encoding the AttP site and the integrase provides for an integration event which is referred to as site-specific integration. DNA containing the AttP site and the integrase gene is capable of integration into a corresponding AttB site of a mycobacterium chromosome.

It is to be understood that the exact DNA sequence encoding the attP site may vary among different phages, and that the exact DNA sequence encoding the attB site may vary among different mycobacteria.

The integration event results in the formation of two new junction sites called AttL and AttR, each of which contain part of each of AttP and AttB. The inserted and integrated non-phage DNA which includes the first DNA sequence and the at least one DNA sequence, which encodes a protein or polypeptide, or fragment or derivative thereof, which includes an epitope which is recognized by cytotoxic T lymphocytes is flanked by the AttL and AttR sites. The insertion and integration of the phage DNA portion results in the formation of a transformed mycobacterium.

The DNA may further include DNA which encodes a selectable marker or markers; or other proteins or polypeptides of interest, such as, but not limited to anti-tumor agents, enzymes, lymphokines, pharmacologic agents, immunopotentiators, and reporter molecules of interest in a diagnostic context.

Selectable markers which may be encoded include, but are not limited to, the kanamycin resistance marker, the neomycin

resistance marker, the chloroamphenicol resistance marker, and the hygromycin resistance marker.

The phage DNA portion of the present invention, which includes the first DNA sequence encoding mycobacterium phage integration into a mycobacterium chromosome, and the at least one DNA sequence encoding a protein or polypeptide, or fragment or derivative thereof, which includes an epitope recognized by cytotoxic T lymphocytes, may be constructed through genetic engineering techniques known to those skilled in the art. In a preferred embodiment, the phage DNA portion may be a plasmid including, in addition to the DNA encoding integration and the DNA encoding a protein or polypeptide, or fragment or derivative thereof, which includes an epitope recognized by cytotoxic T lymphocytes, an origin of replication for any of a wide variety of organisms, which includes, but is not limited to, E.coli, Streptomyces species, Bacillus species, Staphylococcus species, Shigella species, Salmonella species and various species of pneumococci. Most preferably, the plasmid includes an origin of replication for E.coli.

The phage DNA portion also may include a suitable promoter. Suitable promoters include, but are not limited to, mycobacterial promoters and mycobacteriophage promoters such as those hereinabove described.

The promoter sequence may, in one embodiment, be part of an expression cassette which also includes a portion of the gene normally under the control of the promoter, as hereinabove described. For example, when a mycobacterial HSP60 or HSP70 promoter is employed, the expression cassette may include, in addition to the promoter, a portion of the gene for the HSP60 or HSP70 protein. When the expression cassette and the DNA encoding the protein or polypeptide, or fragment or derivative thereof, which includes an epitope which is recognized by cytotoxic T lymphocytes are expressed, the protein expressed by the cassette and the DNA encoding the protein or polypeptide is a fusion

protein of a fragment of a mycobacterial protein (eg., the HSP60 or HSP70 protein), and the protein or polypeptide, or fragment or derivaitve thereof, which includes an epitope which is recognized by cytotoxic T lymphocytes.

In a preferred embodiment, the transcription initiation site, the ribosomal binding site, and the start codon, which provides for the initiation of the translation of mRNA, are each of mycobacterial origin. The stop codon, which stops translation of mRNA, thereby terminating synthesis of the protein or polypeptide, or fragment or deriviatve thereof, which includes an epitope which is recognized by cytotoxic T lymphocytes, and the transcription termination site, may be of mycobacterial origin, or of other bacterial origin, or such stop codon and transcription termination site may be those of the DNA encoding the protein or polypeptide, or fragment or derivative thereof, which includes an epitope which is recognized by cytotoxic T lymphocytes.

Examples of DNA which includes a first DNA sequence which is a phage DNA portion encoding bacteriophage integration into a mycobacterium chromosome, and DNA including the at least one DNA sequence encoding a protein or polypeptide, or fragment or derivative thereof, which includes an epitope which is recognized by cytotoxic T lymphocytes are further described in application Serial No. 553,907, filed July 16, 1990, the contents of which are hereby incorporated by reference.

Mycobacteria which are transformed which DNA which encodes for a protein or polypeptide or fragment(s) or derivative(s) thereof, which includes an epitope which is recognized by cytotoxic T lymphocytes, may be employed in a composition, such as a vaccine, for inducing a CTL response in an aniaml. The vaccine may be administered to a human or non-human animal.

To form such a vaccine, the transformed mycobacteria are administered in conjunction with a suitable pharmaceutical carrier. As representative examples of suitable carriers there

may be mentioned: mineral oil, alum, synthetic polymers, etc. Vehicles for vaccines are well known in the art and the selection of a suitable vehicle is deemed to be within the scope of those skilled in the art from the teachings contained herein. The selection of a suitable vehicle is also dependent upon the manner in which the vaccine is to be administered. The vaccine may be in the form of an injectable dose and may be administered intramuscularly, intravenously, orally, intradermally, or by subcutaneous administration.

Other means for administering the vaccine or therapeutic agent should be apparent to those skilled in the art from the teachings herein; accordingly, the scope of the invention is not to be limited to a particular delivery form.

When the transformed mycobacteria are employed as a vaccine, such a vaccine has important advantages over other presently available vaccines. Mycobacteria have, as hereinabove indicated, adjuvant properties among the best currently known and, therefore, stimulate a recipient's immune system to respond with great effectiveness. This aspect of the vaccine induces cell-mediated immunity and thus is especially useful in providing immunity against pathogens in cases where cell-mediated immunity appears to be critical for resistance. Also, mycobacteria may stimulate long-term memory or immunity. It thus may be possible to prime long-lasting T cell memory, which stimulates secondary antibody responses neutralizing to the infectious agent. Such priming of T cell memory is useful, for example, against pertussis, malaria, influenza virus, Herpes virus, rabies, Rift Valley fever virus, dengue virus, measles virus, Human Immunodeficiency Virus (HIV), and respiratory syncytial virus.

The invention will now be described with respect to the following examples; however, the scope of the present invention is not to be limited thereby.

Example 1

A. Construction of plasmid including mycobacterial promoter expression cassette and lacZ gene.

1. Construction of pYUB125

Plasmid pAL5000, a plasmid which contains an origin of replication of M. fortuitum, and described in Labidi, et al., FEMS Microbiol. Lett., Vol. 30, pgs. 221-225 (1985) and in Gene, Vol. 71, pgs. 315-321 (1988), is subjected to a partial Sau 3A digest, and 5kb fragments are gel purified. A 5kb fragment is then ligated to Bam HI digested pIJ666 (an. E. coli vector containing an E. coli origin of replication and also carries neomycin-kanamycin resistance, as described in Kieser, et al., Gene, Vol. 65, pgs. 83-91 (1988) to form plasmid pYUB12. A schematic of the formation of plasmid pYUB12. A schematic of the formation of plasmid pYUB12 is shown in Figure 1. pYUB12 and pIJ666 were then transformed into M. smegmatis and BCG. Neomycin-resistant transformants that were only obtained by pYUB12 transformation confirmed that pAL5000 conferred autonomous replication to pIJ666 in M. smegmatis and BCG.

Shotgun mutagenesis by Snapper, et al (1988, hereinabove cited) indicated that no more than half of the pAL5000 plasmid was necessary to support plasmid replication in BCG. This segment presumably carried open reading frames ORF1 and ORF2, identified by Rauzier, et al., Gene, Vol. 71, pgs. 315-321 (1988), and also presumably carried a mycobacterial origin of replication. pYUB12 is then digested with HpaI and EcoRV, a 2586 bp carrying this region or segment pAL5000 is removed and ligated to PvuII digested pYUB8. Plasmid pYUB8 (a pBR322 derivative) includes an E. coli replicon and a kan^R (aph) gene. Ligation of the 2586 bp pYUB12 fragment to PvuII digested pYUB8 results in the formation of pYUB53, as depicted in Figure 2. Transformation of pYUB53 confirmed that the EcoRV-HpaI fragment, designated M.rep, was capable of supporting autonomous replication in BCG.

Plasmid pYUB53 was then digested with AatI, EcoRV, and PstI in order to remove the following restriction sites:

AatI 5707
EcoRI 5783
BamHI 5791
SalI 5797
PstI 5803
PstI 7252
SalI 7258
BamHI 7264
EcoRI 7273
ClaI 7298
HindIII 7304; and
EcoRV 7460

Fragment ends are then flushed with T4 DNA polymerase and religated to form plasmid pYUB125, construction of which is shown in Figure 3.

2. Elimination of superfluous vector DNA from pYUB125

792 bases of the tet gene, which had been inactivated by prior manipulations, was eliminated by a complete NarI digest, gel purification of the 6407 bp fragment, and ligation/recirculation, transformation of E. coli strain HB101, and selection of Kan^R transformants. The construction of resulting plasmid, pMV101, is schematically indicated in Figure 4, and the DNA sequence of pMV101, which includes markings of regions which will be deleted, and of mutations, as hereinafter described, is shown in Figure 5.

3. Construction of expression cassette based on BCG HSP60.

Among the most abundant proteins in mycobacteria is the HSP60 heat shock protein (also known as the 65 kda antigen). Because abundance of the HSP60 protein in mycobacteria indicates strong HSP60 gene expression, the sequence controlling HSP60 expression was chosen to control expression of heterologous genes encoding antigens or other proteins in BCG.

The published sequence of the BCG HSP60 gene (Thole, et al, Infect. and Immun., Vol. 55, pgs. 1466-1475 (June 1987)), and

surrounding sequence permitted the construction of a cassette carrying expression control sequences (i.e., promoter and translation initiation sequences) by PCR. The BCG HSP61 cassette (Figure 6) contains 375 bases 5' to the BCG HSP60 start codon, and 15 bases (5 codons) 3' to the start codon. PCR oligonucleotide primers were then synthesized. Primer Xba-HSP60, of the following sequence:

CAG ATC TAG ACG GTG ACC ACA ACG CGC C /

was synthesized for the 5' end of the cassette, and primer Bam-HSP61, of the following sequence:

CTA GGG ATC CGC AAT TGT CTT GGC CAT TG 2

was synthesized for the 3' end of the cassette. The primers were used to amplify the cassette by PCR from BCG strain Pasteur chromosomal DNA. The addition of the Bam HI site at the 3' end of the cassette adds one codon (Asp) to the first six codons of the HSP60 gene.

Each of pMV101 and the PCR cassette HSP61 was digested with NheI and BamHI. The PCR cassette was then inserted between the NheI and BamHI sites of pMV101, then ligated to form plasmid pMV65A (Figure 7).

The E. coli lac Z gene (Figure 8) was used as a reporter, or marker gene to assay the ability of the HSP61 cassette to express heterologous genes in BCG. A BamHI restriction fragment carrying the lac Z gene was cloned into the Bam HI site of Bam HI digested pMV65A, resulting in the formation of pMV65A/LZ as indicated schematically in Figure 7. The formation of pMV65A/LZ results in a fusion between the HSP60 and lac Z genes at the sixth codon of the HSP60 gene and the sixth codon of the lac Z gene. pMV65A/LZ was then transformed into E. coli. Blue E. coli colonies were selected on x-gal plates for the presence of pMV65A/LZ, thus indicating that the HSP60 promoter and translation initiation sequences were also active in E. coli.

pMV65A/LZ was then transformed into BCG and plated on Dubos Oleic Agar plates containing x-gal. All BCG colonies resulting

from this transformation exhibited blue color, thus indicating that the lac Z gene product (B-galactosidase) was expressed in BCG. SDS polyacrylamide gel electrophoresis was performed on lysates of the pMV65A/LZ BCG recombinants, revealing that B-galactosidase protein was expressed to levels in excess of 10% of total BCG protein (as determined by staining with Coomassie brilliant blue). These data indicated that BCG HSP61 expression cassette was functional in expression vector pMV65A.

Example 2

Cytotoxic T lymphocyte response to E. coli β-galactosidase.

E. coli β-galactosidase was expressed in BCG as a six amino acid fusion protein with BCG hsp 60 protein using extrachromosomal plasmid vector pMV65A/LZ utilizing the HSP60 promoter to drive expression. The recombinant BCG was grown to mid-log phase in Dubos media and concentrated by centrifugation. The bacteria were then re-suspended in PBS plus 0.05% Tween 80 and cup sonicated briefly to disperse clumped bacteria. Six week old BALB/c mice were inoculated with a single dose of 2×10^2, 2×10^4, or 2×10^6 colony forming units (CFU'S determined post-inoculation) by either intradermal (ID), intraperitoneal (IP), or intravenous (IV) injection. At 14 or 19 weeks post-immunization splenocytes were harvested from mice and CTL activity was measured. CTL activity was measured as follows:

Splenocytes (ACK-treated, 5×10^6/ml) were stimulated in vitro in 10 ml in upright T25 flasks by co-culture for 5 days with mitomycin C-treated cells transfected with the lac Z gene (C3-4 cells; 5×10^6/ml). A 4 hr. ^{51}Cr release assay was then performed in triplicate using P815 and P13.1 cells (P815 cells transfected with the lac Z gene) as targets. Various effector-target ratios were tested using 5,000 targets/well. Specific lysis was calculated as follows: % specific lysis = 100 X [release by effector cells minus spontaneous release/ maximal release minus spontaneous release].

At 19 weeks post-immunization, the remaining animals in each group were boosted by intraperitoneal injection at 10 µg of purified lac Z emulsified in incomplete Freund's adjuvant (IFA). Splenocytes were then harvested from these animals at 23 weeks and CTL activity was again measured. Unimmunized animals or animals immunized with lac Z emulsified in IFA, or animals immunized with vaccinia virus expressing lac Z served as controls.

The results of the avove experiments, as determined by % specific lysis of target cells, indicated that a CTL response was induced in mice immunized with BCG transformed with the expression vector pMV65A/LZ.

Example 3

Construction of integrating plasmid including mycobacterial promoter expression cassette and HIV-I-gp 120 gene.

1. Elimination of undesirable restriction sites in aph (kan^R) gene.

To facilitate future manipulations, the HindIII and ClaI restriction sites in the aph gene in plasmid pMV101 were mutagenized simultaneously by polymerase chain reaction (PCR) mutagenesis according to the procedure described in Gene, Vol. 77 pgs. 57-59 (1989). The bases changed in the aph gene were at the third position of codons (wobble bases) within each restriction site and the base substitutions made were designed not to change the amino acid sequence of the encoded protein.

Separate PCR reactions of plasmid pMV101 with primer ClaMut-Kan + HindRMut-Kan and HindFMut-Kan + Bam-Kan were performed at 90°C (1 min.), 50°C (1 min.), and 72°C (1 min.) for 25 cycles. The PCR primers had the following base seqeuences:

ClaMut-Kan

CTT GTA TGG GAA GCC CC 3

HindRMut-Kan

GTG AGA ATG GCA AAA GAT TAT GCA TTT CTT TCC AG 5

HindFMut-Kan

GTG TGG AAA GAA ATG CAT AAT CTT TTG CCA TTC TCA CCG G 5'

Bam-Kan

CGT AGA GGA TCC AGA GGA CG 6

The resulting PCR products were gel purified and mixed and a single PCR reaction without primers was performed at 94°C (1 min.), 72°C (1 min.) for 10 cycles. Primers ClaMut-Kan and Bam-Kan were added and PCR was resumed at 94°C (1 min.), 50°C (1 min.), and 72°C (2 min.) for 20 cylces. The resulting PCR product (Kan. mut) was digested with BamHI and gel purified. Plasmid pMV101 was digested with ClaI and cohesive ends were filled in by Klenow + dCTP + dGTP. Klenow was heat inactivated and the digest was further digested with BamHI. The 5232 base pair fragment was gel purified and mixed with fragment Kan.mut and ligated. The ligation was transformed into E. coli strain HB101 and Kan^R colonies were screened for plasmids resistant to ClaI and HindIII digestion. Such plasmids were designated as pMV110, which is depicted in Figure 4.

2. Elimination of sequences not necessary for plasmid replication in mycobacteria.

Plasmid pMV110 was resected in separate constructions to yield plasmids pMV111 and pMV112. In one construction, pMV110 was digested with NarI and BalI, the ends were filled in, and a 5296 base pair fragment was ligated and recircularized to form pMV111. In another construct, pMV110 was digested with NdeI and SplI, the ends were filled in, and a 5763 base pair fragment was ligated and recircularized to form pMV112. Schematics of the constructions of pMV111 and pMV112 are shown in Figure 9. These constructions further eliminated superfluous E. coli vector sequences derived from pAL5000 not necessary for mycobacterial replication. Cloning was performed in E. coli. Plasmids pMV111 and pMV112 were tested for the ability to replicate in M. smegmatis. Because both plasmids replicated in M. smegmatis the deletions of each plasmid were combined to construct pMV113. (Figure 9).

To construct pMV113, pMV111 was digested with BamHI and EcoRI, and a 1071 bp fragment was isolated. pMV112 was digested with BamHI and EcoRI, and a 3570 bp fragment was isolated, and then ligated to the 1071 bp fragment obtained from pMV111 to form pMV113. These constructions thus defined the region of pAL5000 necessary for autonomous replication in mycobacteria as no larger than 1910 base pairs.

3.. Mutagenesis of restriction sites in mycobacterial replicon.

To facilitate further manipulations of the mycobacterial replicon, PCR mutagenesis was performed as above to eliminate the Sal I, EcoRI, and BglII sites located in the open reading frame known as ORF1 of pAL5000. PCR mutagenesis was performed at wobble bases within each restriction site and the base substitutions were designed not to change the amino acid sequence of the putative encoded ORF1 protein. The restriction sites were eliminated one at a time for testing in mycobacteria. It was possible to eliminate the SalI and EcoRI without altering replication in M. smegmatis. In one construction PCR mutagenesis was performed at EcoRI1071 of pMV113 with primers Eco Mut - M.rep and Bam-M.rep to form pMV117, which lacks the EcoRI1071 site. Primer Eco Mut - M.rep has the following sequence:

TCC GTG CAA CGA GTG TCC CGG A; 7

and Bam-M.rep has the following sequence:

CAC CCG TCC TGT GGA TCC TCT AC. 8

In another construction, PCR mutagenesis was performed at the SalI 1389 site with primer Sal Mut - M.rep and Bam-M.rep to form pMV119, which lacks the SalI 1389 site. Primer Sal Mut-M.rep has the following sequence:

TGG CGA CCG CAG TTA CTC AGG CCT. 9

pMV117 was then digested with ApaLI and BglII, and a 3360 bp fragment was isolated. pMV119 was digested with ApaLI and BglII, and a 1281 bp fragment was isolated and ligated to the 3360 bp fragment isolated from pMV117 to form pMV123. A schematic of the

constructions of plasmids pMV117, pMV119, and pMV123 is shown in Figure 10. Elimination of the BglII site, however, either by PCR mutagenesis or Klenow fill in, eliminated plasmid replication in mycobacteria, thus suggesting that the BglII site is in proximity to, or within a sequence necessary for mycobacteria plasmid replication.

4. Construction of pMV200 series vectors.

To facilitate manipulations of all the components necessary for plasmid replication in E. coli and mycobacteria, (E. rep. and M. rep.) and selection of recombinants (Kan^R), cassettes of each component were constructed for simplified assembly in future vectors and to include a multiple cloning site (MCS) containing unique restriction sites and transcription and translation terminators. The cassettes were constructed to allow directional cloning and assembly into a plasmid where all transcription is unidirectional.

Kan^R Cassette

A DNA cassette containing the aph (Kan^R) gene was constructed by PCR with primers Kan5' and Kan3'. An SpeI site was added to the 5' end of the PCR primer Kan3', resulting in the formation of a PCR primer having the following sequence:

CTC GAC TAG TGA GGT CTG CCT CGT GAA G. 10

Bam HI + NheI sites were added to the 5' end of the primer Kan5', resulting in the formation of a PCR primer having the following sequence:

CAG AGG ATC CTT AGC TAG CCA CT GAC GTC GGG G. //

PCR was performed at bases 3375 and 4585 of pMV123, and BamHI and NheI sites were added at base 3159, and an SpeI site was added at base 4585. Digestion with BamHI and SpeI, followed by purification resulted in a 1228/2443 Kan^R cassette bounded by BamHI and SpeI cohesive ends with the direction of transcription for the aph gene proceeding from BamHI to Spe I.

E. rep. cassette

A DNA cassette containing the ColEI replicon of pUC19 was constructed by PCR with primers E.rep/Spe and E.rep/Mlu. An SpeI site was added to the 5' end of PCR primer E.rep/Spe and an MluI site was added to the 5' end of PCR primer E.rep./Mlu. The resulting primers had the following sequences:

E.rep./Spe

CCA CTA GTT CCA CTG AGC GTC AGA CCC 12

E.rep./Mlu

GAC AAC GCG TTG CGC TCG GTC GTT CGG CTG. 13

PCR was performed at bases 713 and 1500 of pUC19, and an MluI site was added to base 713, and a SpeI site was added to base 1500. Digestion with MluI and SpeI, followed by purification resulted in an E.rep. cassette bounded by SpeI and MluI cohesive ends with the direction of transcription for RNA I and RNA II replication primers proceeding from SpeI to MluI.

M.rep. cassette

A DNA cassette containing sequences necessary for plasmid replication in mycobacteria was constructed by PCR of pMV123 with primers M.rep/Mlu and M.rep/Bam. An MluI site was added to the 5' end of PCR primer M.rep/Mlu. A BamHI site was added to the 5' end of PCR primer M.rep/Bam. The resulting PCR primers had the following base sequences:

M.rep./Mlu

CCA TAC GCG TGA GCC CAC CAG CTC CG 14

M.rep./Bam

CAC CCG TCC TGT GGA TCC TCT AC 15

PCR was performed at bases 134 and 2082 of pMV123. An MluI sited was added to base 2082. Digestion with BamHI and MluI, followed by gel purification resulted in a 1935 base pair DNA cassette bounded by MluI and BamHI cohesive ends with the direction of transcription for the pAL5000 ORF1 and ORF2 genes proceeding from MluI to Bam HI.

The Kan^R, E.rep, and M.rep PCR cassettes were then mixed in equimolar concentrations and ligated, and then transformed in E.

coli strain HB101 for selection of Kan^R transformants. Colonies were screened for the presence of plasmids carrying all three cassettes after digestion with BamHI + MluI + SpeI and designated pMV200. An additional restriction site, NcoI, was eliminated from the M.rep cassette by digestion of pMV200 with NcoI, fill in with Klenow, and ligation and recircularization, resulting in the formation of pMV201. A schematic of the formation of pMV200 from pMV123 and pUC19, and of pMV201 from pMV200, is shown in Figure 11. Plasmids pMV200 and pMV201 were transformed into M. smegmatis and BCG. Both plasmids yielded Kan^R transformants, thus indicating their ability to replicate in mycobacteria.

A synthetic multiple cloning sequence (MCS) (Figure 12) was then designed and synthesized to facilitate versatile molecular cloning and manipulations for foreign gene expressions in mycobacteria, and for integration into the mycobacterial chromosome. The synthetic MCS, shown in Figure 12, contains 16 restriction sites unique to pMV201 and includes a region carrying translation stop codons in each of three reading frames, and a transcription terminator derived from E. coli 5S ribosomal RNA (T1).

To insert the MCS cassette, pMV201 was digested with NarI and NheI, and the resulting fragment was gel purified. The MCS was digested with HinPI and NheI and, the resulting fragment was gel purified. The two fragments were then ligated to yield pMV204. A schematic of the construction of pMV204 is shown in Figure 13.

Plasmid pMV204 was then further manipulated to facilitate removal of the M.rep cassette in further constructions. pMV204 was digested with MluI, and an MluI - Not I linker was inserted into the MluI site between the M.rep and the E.rep to generate pMV206. A schematic of the construction of pMV206 from pMV204 is shown in Figure 14, and the DNA sequence of pMV206 is given in Figure 15.

5. Construction of expression cassette based on BCG HSP60.

The HSP61 cassette (Figure 6) was constructed as hereinabove described in Example 1.

Each of pMV206 and the PCR cassette HSP61 was digested with XbaI and BamHI. The PCR cassette was then inserted between the XbaI and BamHI sites of pMV206, then ligated to form plasmid pMV261. The construction of this plasmid is shown schematically in Figure 17. The reading frame and the restriction sites of the multiple cloning site of pMV261 is shown in Figure 16.

The E. coli lac Z gene was used as a reporter, or marker gene to assay the ability of the HSP61 cassette to express heterologous genes in BCG. A BamHI restriction fragment carrying the lac Z gene was cloned into the Bam HI site of Bam HI digested pMV261, resulting in the formation of pMV261/LZ. A schematic of the construction of pMV261/LZ is shown in Figure 18. The formation of pMV261/LZ results in a fusion between the HSP60 and lac Z genes at the sixth codon of the HSP60 gene and the sixth codon of the lac Z gene. pMV261/LZ was then transformed into E. coli. Blue E. coli colonies were selected on x-gal plates for the presence of pMV261/LZ, thus indicating that the HSP60 promoter and translation initiation sequences were also active in E. coli.

pMV261/LZ was then transformed into BCG and plated on Dubos Oleic Agar plates containing x-gal. All BCG colonies resulting from this transformation exhibited blue color, thus indicating that the lac Z gene product (B-galactosidase) was expressed in BCG. SDS polyacrylamide gel electrophoresis was performed on lysates of the pMV261/LZ BCG recobinants, revealing that B-galactosidase protein was expressed to levels in excess of 10% of total BCG protein (as determined by staining with Coomassie brilliant blue). These data indicated that BCG HSP61 expression cassette was functional in expression vector pMV261.

Plasmid pMV261/LZ was then shown to replicate autonomously, and express the E. coli B-galactosidase, or lacZ gene, driven by the BCG promoter HSP60, in M. smegmatis and BCG.

6. Transfer of mycobacteriophage L5 integration sequences to BCG expression vector.

Plasmid pMH9.4, which includes the mycobacteriophage L5 attP site, and the L5 integrase gene, was employed in providing the L5 integration sequences to a BCG expression vector. The construction of pMH9.4, as well as its integration into M. smegmatis and BCG, is described below in sections (i) through (vi).

(i) Identification of the DNA sequences of the attachment sites, attB, attL, and attR, of M.smegmatis.

Using standard technologies, a lambda EMBL3 library was constructed using chromosomal DNA prepared from $mc^2$61 (a strain of M. smegmatis which includes an M. smegmatis chromosome into which has been integrated the genome of mycobacterial phage L5) and digested with Bam HI. Phage L5 contains DNA having restriction sites identical to those of phage L1 (Snapper, et al. 1988), except that L5 is able to replicate at 42°C and phage L1 is incapable of such growth. This library was then probed with a 6.7 kb DNA fragment isolated from the L5 genome that had been previously identified as carrying the attP sequence (Snapper, et al 1988). One of the positive clones was plaque purified, DNA prepared, and a 1.1 kb Sal I fragment (containing the AttL sequence) sub-cloned into sequencing vector pUC119. The DNA sequence of this fragment was determined using a shotgun approach coupled with Sanger sequencing. By isolating and sequencing the attL junction site and comparing this to the DNA sequence of L5 that was available, a region was determined where the two sequences aligned but with a specific discontinuity present. The discontinuity represents one side of a core sequence, which is identical in AttP, attB, and attL. The region containing the recombinational crossover point is shown in Figure 19.

The attL DNA (1.1 kb Sal I fragment) was used as a probe to hybridize to a Southern blot of Bam HI digested $mc^2$6 DNA, which

is a strain of M. smegmatis which includes an M. smegmatis chromosome without any phage integration (Jacobs, et al, 1987, hereinabove cited.). A single band of approximately 6.4 kb was detected corresponding to the attB sequence of M. smegmatis. This same attL probe was used to screen a cosmid library of $mc^2 6$ (provided by Dr. Bill Jacobs of the Albert Einstein College of Medicine of Yeshiva University), and a number of positive cosmid clones were identified. DNA was prepared from these clones, and a 1.9 kb Sal I fragment (containing the attB site) that hybridizes to the attL probe was subcloned into pUC119 for sequencing and further analysis. The DNA sequence containing the core sequence was determined and is shown in Figure 19. The core sequence, which is identical in attP, attB and attL, has a length of 43bp.

The $mc^2 61$ lambda EMBL3 library was then probed with the 1.9kb SalI fragment containing the attB site. Positive plaques were identified, DNA was prepared, and analyzed by restriction analysis and Southern blots. Lambda clones were identified that contained a 3.2kb Bam HI fragment containing the putative attR site. The 3.2kb Bam HI fragment was purified and cloned into pUC119 for sequencing and further analysis.

(ii) Determination of attP-integrase region of L5 genome.

Concurrent with the above procedures, a significant portion of the DNA sequence of L5 had been determined and represented in several "contigs" or islands of DNA sequence. Sequences of the 6.7kb Bam HI fragment hereinabove described were determined by (a) analysis of the location of Bam HI sites in the contigs of the DNA of L5, and (b) by determining a short stretch of DNA sequence from around the Bam HI sites of plasmid pJR-1 (Figure 24), which carries the 6.7kb Bam HI fragment of L5.

A segment of DNA sequence was located that represented the 6.7kb Bam HI fragment of phage L5. Studies of other phages have shown that the integrase genes are often located close to the attP site. It was thus determined that the L5 integrase (int)

gene should lie either within the 6.7kb Bam HI fragment or in a DNA sequence on either side of it. The DNA sequence in the regions was then analyzed by translating it into all six possible reading frames and searching these amino acid sequences for similarity to the family of integrase related proteins, and through computer-assisted analysis of the DNA sequence. As shown in Figure 20, there are shown two domains of reasonably good conservation among L5 integrase and other integrases, and three amino acid residues that are absolutely conserved in domain 2. (See Yagil, et al., J. Mol. Biol., Vol. 207, pgs. 695-717 (1989), and Poyart-Salmeron, et al., J. EMBO., Vol. 8, pgs. 2425-2433 (1989)). A region was identified, and analysis of the corresponding DNA sequence showed a reading frame that could encode for a protein of approximately 333 amino acids. These observations identified the putative int gene.

The location of the int gene was not within the 6.7kb Bam HI fragment; however, it was very close to it with one of the Bam HI sites (that defines the 6.7kb Bam HI fragment) less than 100 bp upstream of the start of the gene. Analysis of the Bam HI sites showed that the int gene lay within a 1.9kb Bam HI fragment located adjacent to the 6.7kb Bam HI fragment. This 1.9kb Bam HI fragment was cloned by purification of the fragment from a Bam HI digest of L5 DNA and cloning into pUC 119, to generate pMH1 (Figure 25).

From a combination of the above approaches, a schematic of the organization of the attP-int region of L5 was constructed (Figure 26), and the gene sequence of the attP-int region is given in Figure 22.

(iii) Construction of pMH5.

The 6.7kb Bam HI fragment of mycobacteriophage L5, which contains the attP site, as hereinabove described, was cloned into the Bam HI site of pUC 119 (Figure 23). This was achieved by purifying the 6.7kb Bam HI fragment from a Bam HI digest of L5

DNA separated by agarose gel electrophoresis and ligating with Bam HI cut pUC 119. DNA was prepared from candidate recombinants and characterized by restriction enzyme analysis and gel electrophoresis. A recombinant was identified that contained the 6.7kb Bam HI fragment of L5 cloned into pUC 119. This plasmid was named pJR-1, as shown in Figure 24.

Analysis of DNA sequence data from a project to sequence L5 showed that a 1.9kb Bam HI fragment adjacent to the 6.7kb Bam HI fragment hereinabove described contained the integrase gene.

A plasmid containing a 1.9kb Bam HI fragment containing the DNA encoding for the integrase cloned into the Bam HI site of pUC 119 was constructed. The 1.9kb fragment was purified from a Bam HI digest of L5 DNA and cloned into the Bam HI site of pUC 119. Construction of the recombinant was determined by restriction analysis and gel electrophoresis. This plasmid was called pMH1, the construction of which is shown schematically in Figure 25.

pJR-1 was then modified by digestion with EcoRI and SnaBI (both are unique cloning sites), between which is a Bam HI site. The EcoRI-SnaBI fragment, including the Bam HI site was excised, and the plasmid was religated to form plasmid of pMH2, which contains on Bam HI site compared to two Bam HI sites contained in pJR-1. A schematic of the construction of pMH2 is shown in Figure 26.

The 1.9kb Bam HI fragment, which includes the integrase gene, was purified from a Bam HI digest of pMH1 and ligated to Bam HI digested pMH2. Recombinants were identified as above and the orientation of the 1.9kb fragment determined. A plasmid called pMH4 was thus constructed (Figure 27) in which the region from the Sna BI site (upstream of attP) through to the Bam HI site (downstream of the integrase gene) was identical to that in L5.

pMH4 was digested with HindIII (unique site) and was ligated to a 1kb HindIII fragment purified from pKD43 (supplied by Keith Darbyshire of the Nigel Gindley Laboratory) that contains the

gene determining resistance to kanamycin. Recombinants were identified and characterized as above. This plasmid is called pMH5. A schematic of the construction of pMH5 is shown in Figure 28.

(iv) Integration of pMH5 into attB of M. smegmatis.

Plasmids pYUB12 (a gift from Dr. Bill Jacobs, a schematic of the formation of which is shown in Figure 1), pMD01 (Figure 29), and pMH5 were electroporated, with four different concentrations of plasmid DNA over a 1,000-fold range, into M. smegmatis strain mc^2155, a strain which is able to support plasmid replication. In sections (iv) through (vi), all electroporation procedures of M. smegmatis, or of BCG, were carried out as follows:

Cultures of organism were grown in Middlebrook 7H9 media, as described by Snapper, et al. (1988), harvested by centrifugation, washed three times with cold 10% glycerol, and resuspended at approximately a 100 x concentration of cells.

1 µl of DNA was added to 100 µl of cells in an ice-cold cuvette and pulsed in a Bio-Rad Gene Pulser, and given a single pulse at 1.25 kv at 25 µF. 1 ml of broth was added the cells incubated for 1 hr. at 37°C for expression of the antibiotic-resistant marker. Cells were then concentrated and plated out on Middlebrook or tryptic soy media containing 15 µg/ml kanamycin. Colonies were observed after 3 to 5 days incubation at 37°C.

Each of pYUB12, pMD01, and pMH5 carries kanamycin resistance. Plasmid pYUB12 carries an origin of DNA replication, while pMD01 lacks a mycobacterial origin of replication. Plasmid pMH5 does not carry a mycobacterial origin of replication, but carries a 2kb region of phage L5 which contains the attP site and the integrase gene (Figure 22). The number of transformants were linear with DNA concentration. Plasmid pYUB12 gives a large number of transformants (2×10^5 per µg DNA) in mc^2155, while pMH5 gives 6×10^4 transformants per µg DNA, and pMD01 gives no transformants.

The above experiment was then repeated by electroporating the plasmids pYUB12, pMD01, and pMH5 into M. smegmatis strain $mc^2 6$, which does not support plasmid replication. No transformants in $mc^2 6$ were obtained from pYUB12 or pMD01, while pMH5 gave approximately 10^4 kanamycin resistant transformants in $mc^2 6$ per µg of DNA, thus indicating integration of pMH5 into the $mc^2 6$ chromosome.

DNA from six independent pMH5 transformants (four in mc^2 155 and two in $mc^2 6$) was prepared. These DNA's (along with DNA from both $mc^2 155$ itself, and $mc^2 155$ carrying the plasmid pYUB12) were digested with a restriction enzyme, and analyzed by Southern blot and hybridization with the M. smegmatis 1.9kb attB probe hereinabove described. As shown in Figure 30, all six transformants have integrated into the attB site, resulting in the production of two new DNA fragments with different mobilities. If pMH5 did not integrate into the attB site, it would be expected that a single band, corresponding to the attB site in the $mc^2 155$ control, would be obtained.

(v) Construction of pMH9.2 and pMH9.4

pUC119 was digested with HindIII, and a 1kb HindIII fragment, containing a kanamycin resistance gene, purified from pKD43, was ligated to the HindIII digested pUC119 to form pMH8 (Figure 31). A 2kb SalI fragment (bp 3226-5310), which carries the attP and integrase gene from SalI digested pMH5, was purified and inserted in both orientations relative to the vector backbone of SalI digested pMH8 to form plasmids pMH9.2 and pMH9.4 (Figures 32 and 33).

M. smegmatis strain $mc^2 155$ cells carrying, as a result of electroporation, plasmid pYUB12, pMH9.2 or pMH9.4, or strain $mc^2 6$ cells carrying plasmid pMH5, as a result of electroporation as hereinabove described, were grown to saturation in broth with kananmycin. Cultures were then diluted 1:100 into broth without kanamycin and grown to saturation. Two further cycles of dilution and growth were done, corresponding to about 20

generations of bacterial growth. Cultures were plated out to single colonies on non-selective plates, and approximately 100 of these colonies were patch plated onto both non-selective and selective plates. The % of colonies that were sensitive to kanamycin, thus corresponding to the percentage of cells which lost the plasmid, is given below in Table I.

Table I

	% loss
pYUB12 (mc^2155)	35
pMH5 (mc^26)	17
pMH9.2 (mc^2155)	3
pMH9.4 (mc^2155)	0

(vi) Transformation of BCG with pMH9.4

The 1.9 kb Sal I fragment, which includes the M. smegmatis attB site as hereinabove described was cloned into pUC119, and the plasmid generated was named pMH-12. (Figure 34).

Gel purified Sal I 1.9kb M. smegmatis fragment containing attB (isolated from pMH-12) was used to probe a Southern transfer of Bam HI digested mycobacterial DNA's, including BCG substrain Pasteur, shown in Figure 35. This demonstrated that there is one Bam HI fragment of BCG that strongly hybridizes to the M. smegmatis attB probe and three hybridize weakly. The strongest hybridizing band is the fastest moving band (approximately 1.9 kb).

The same probe as above was used to probe a BCG cosmid library (provided by Dr. Bill Jacobs) and positive clones were identified. DNA was prepared from several positive clones and analyzed by restriction analysis and Southern blotting. The 1.9 kb Bam HI fragment (corresponding to the strongly hybridizing band in the Southern blot was identified, gel purified from the cosmid DNA and cloned into pUC119. The resulting plasmid was named pMH-15. (Figure 36).

Plasmid pMH-5 and pMH9.4 were electroporated into BCG Pasteur. It was observed that pMH9.4 transforms BCG with high

efficiency (approximately 10^4 transformants/μg DNA), while pMH-5 transforms BCG at low efficiency (1-10 transformants/μg DNA). DNA was prepared from BCG transformants and analyzed by Bam HI restriction and Southern blot analysis, probing with gel purified 1.9kb Bam HI BCG attB fragment from pMH-15. These data are shown in Figure 37 and show that integration of both pMH5 and pMH9.4 is specific to the BCG attB site (ie. the strongly cross-hybridizing fragment in BCG). This is illustrated by the loss of the 1.9kb Bam HI fragment from the transformants and the appearance of two new bands representing attL and attR junction fragments. Figure 37 shows just one of the pMH5/BCG transformants, although all of the four that were analyzed show that one of the bands (the largest) is smaller than expected (and different in each of the transformants), indicating that the transformation effiency of pMH-5 is low in BCG. In contrast, the four pMH9.4 transformants are identical to each other (Figure 37) and give attR and attL junction fragments of the predicted sizes.

Plasmid pMH9.4, which includes the mycobacterial phage L5 attP site and the L5 integrase gene, was digested to completion with either KpnI + PvuII or XbaI + PvuII, and a restriction fragment of 1862 or 1847 base pairs, respectively, each of which contain the attP site and the integrase gene, were purified by agarose gel electrophoresis. Plasmid pMV261/LZ was digested with XbaI or DraI to generate either a 7569 bp or 7574 bp vector fragment. The 7569 bp fragment was ligated to the 1862 bp fragment derived from pMH9.4 to form pMV460/LZ. The 7574 bp fragment was ligated to the 1847 bp fragment derived from pMH9.4 to form pMV460 R/LZ. Plasmids pMV460 F/LZ and pMV460R/LZ each include a mycobacterial replicon, the L5 attP site, and the L5 integrase gene. A schematic of the formation of plasmids pMV460 F/LZ and pMV460R/LZ is shown in Figure 38. To generate derivatives without the mycobacterial plasmid replicon, plasmids pMV460/LZ and pMV460/LZ were digested with NotI and recircularized by ligation to generate pMV360F/LZ and pMV360R/LZ.

A schematic of the construction of pMV360F/LZ and pMV360R/LZ is shown in Figure 39.

Plasmids pMH9.4, pMV261/LZ, pMV460/LZ, pMV460/LZ, pMV460/LZ, and pMV460/LZ were then transformed into M. smegmatis and BCG to test their ability to replicate autonomously or integrate into the M. smegmatis or the BCG chromosome. Transformation with pMH9.4, pMV261/LZ, pMV360F/LZ, and pMV360R/LZ yielded kananmycin resistant transformants of M. smegmatis and BCG. Transformants of pMV261LZ, pMV360F/LZ, and pMV360R/LZ were shown to express E. coli B-galactosidase by SDS-polyacrylamide gel electrophoresis and X-gal assay. Plasmids pMV460F/LZ and pMV460R/LZ failed to yield kanamycin resistant transformants, thus indicating that chromosomal integration of a plasmid carrying sequences mediating autonomous replication is lethal to mycobacteria.

7. Construction of pMV307.

Plasmid pMV206 was digested with NotI to remove the mycobacterial replicon. The resulting 2209 bp fragment, which includes the aph (Kan^R) gene, the E. coli replicon and the multiple cloning site, was ligated and recircularized to form pMV205, the construction of which is schematically depicted in Figure 14.

PCR with primers XbaI-Att/Int and NheI-Att/Int was then performed on a Sal I fragment from pMH9.4, which contains the attP site and the L5 integrase gene. The resulting cassette was then digested with XbaI and NheI and a 1789 bp fragment was gel purified. pMV205 was then digested with NheI, and the resulting fragment was ligated to the 1989 bp fragment obtained from pMH9.4 to form pMV307. A schematic of the construction of pMV307 is shown in Figure 40.

8. Construction of pMV261/HIV1-gp 120.

An SmaI-ClaI antigen gene fragment, or cassette, was constructed by PCR, and cloned between the Bam HI and ClaI restriction sites of pMV261 to form pMV261/HIV1-gp 120.

Plasmid pMV261/HIV1-gp 120 was transformed into BCG, and the presence of the corresponding antigen in BCG was verified by the appearance of immunoreactive protein bands in Western blot analysis of BCG recombinant lysates.

9. Construction of pMV361/HIV1-gp 120

The HIV1-gp 120 antigen gene expression cassette, which includes a promoter sequence and an HIV1-gp 120 gene sequence, was excised from the pMV261 derivatives with NotI and a second restriction enzyme site (Pvu II, Eco RI, Sal I, Cla I or Hind III) and cloned into the integrating plasmid pMV307 between the NotI site and a second enzyme site (Pvu II, Eco RI, Sal I, Cla I or Hind III) to form the plasmid pMV361/HIV-Igp120. The backbone of this plasmid is shown in Figure 41.

Plasmid pMV361/HIV-Igp120 was transformed into BCG and shown to express the corresponding antigens by Western blot analysis (Figure 42) with the appropriate antigen-specific human sera.

Example 4

Cytotoxic T lymphocyte responses to HIV-1-gp 120

HIV-1 gp 120 was expressed in BCG as a six amino acid fusion protein with BCG hsp 60 protein using vector pMV361/HIV-1-gp120, using the hsp 60 promoter to control expression.

Two groups of mice were inoculated with 1 X 10^6 CFU's of recombinant BCG expressing the gp 120 gene from the integrative plasmid pMV361/HIV-1-gp120. One group received the BCG via intraperitonal injection (100 ul) whereas the other group received the BCG by deposition of the dose (10 ul) rubbed into a tail scratch.

CTL activity was measured at various times after immunization. CTL activity was measured as follows:

Two mice from each group were sacrificed at various times after immunation, and the spleens were removed. Single cell suspensions were made and the red blood cells were lyzed with ammonium chloride. The cells were stimulated in vitro for 5 days with P815 cells that were pulsed with peptide P18, a fifteen

residue synthetic peptide within HIV1-gp 120. A 4-hour chromium (Cr^{51}) release assay was then carried out using untreated P815 and peptide P18 pulsed P815 cells as targets. Significant P18-specific CTL activity was observed in the mice immunized by tail scratch 14 weeks after immunization. At 16 weeks, CTL activity was observed in both groups of mice. Upon repeat of this experiement, CTL activity was observed at time points as early as 8 weeks after immunization.

Example 5

Recombinant BCG transformed with pMV361/HIV1-gp 120 were grown to mid-log phase in Dubos media and concentrated by centrifugation. The bacteria were then resuspended in 15% glycerol and frozen using a rate contolled freezing apparatus. The bacteria were stored at -70°C until use (referred to as "vaccine"). A second preparation grown in the same way was not frozen and is referred to as a "fresh" preparation. Prior to immunization of animals, the bacteria were resuspended in PBS + 0.05% Tween 80 to the desired concentration and cup sonicated briefly to disperse clumped bacteria. Six week old BALB/c mice were inoculated with a single dose of 5×10^4 cfu fresh bacteria (determined post inoculation) or 1.5×10^5 frozen bacteria (determined pre-inoculation) by tail scratch (t.s.) injection. At 8 weeks post-immunization, splenocytes were harvested from animals and CTL activity was measured (described below). Splenocytes from unimmunized animals were used as controls in the CTL assays.

CTL activity was determined as follows:

Splenocytes (ACK-treated, 5×10^6/ml) were stimulated in vitro in 10 ml in upright T25 flasks by co-culture for 5 days with mitomycin C-treated P815 cells (5×10^6/ml) that were pulsed with 250 ug/ml of peptide P18 for one hour. A 4 hr. ^{51}Cr release assay was subsequently performed in triplicate using P815 targets with or without pulsing for 1 hour with 250 ug/ml peptide P18. Various effector-target ratios were tested using 5000

targets/well. Specific lysis was calculated as follows: % specific lysis = 100 X [release by effector cells minus spontaneous release/maximal release minus spontaneious release].

The results are given in Figure 42.

As shown in Figure 43, both groups of mice showed an increased CTL response at 8 weeks after immunization as compared with unimmunized mice.

Example 6

Recombinant BCG transformed with pMV361/HIV1-gp 120. were grown to mid-log phase in Dubos media and concentrated by centrifugation. The bacteria were then resuspended in 15% glycerol and frozen using a rate contolled freezing apparatus. The bacteria were stored at -70°C until use (referred to as "vaccine"). A second preparation grown in the same way was not frozen and is referred to as a "fresh" preparation. Prior to immunization of animals, the bacteria were resuspended in PBS + 0.05% Tween 80 to the desired concentration and cup sonicated briefly to disperse clumped bacteria. Six week old BALB/c mice were inoculated with a single dose of 5×10^4 cfu fresh bacteria (determined post inoculation) or 1.5×10^5 frozen bacteria (determined pre-inoculation) by tail scratch (t.s.) injection. At 8 weeks post-immunization, splenocytes were harvested from animals and CTL activity was measured (described below).

CTL activity was determined as follows:

Lymph node cells (5×10^6/ml) were stimulated in vitro in 10 ml in upright T25 flasks by co-culture for 5 days with mitomycin C-treated P815 cells (5×10^6/ml) that were pulsed with 250 ug/ml of peptide P18 for one hour. A 4 hr ^{51}Cr release assay was subsequently performed in triplicate using P815 (matched) or EL4 (mismatched) targets with or without pulsing for 1 hour with 250 ug/ml peptide P18. Various effector target ratios were tested using 5000 targets/well. Specific lysis was calculated as follows: % specific lysis = 100 x [release by effector cells

minus spontaneous release/maximal release minus spontaneous release].

The results of this assay are given in Figure 44.

As shown in Figure 44, a CTL response to HIV-1 gp 120 using lymph node cells was demonstrated following immunization of mice with BCG transformed with pMV361/HIV-1 gp 120.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.

WHAT IS CLAIMED IS:

1. A method of inducing a CTL response in an animal comprising:

administering to an animal mycobacteria transformed with at least one DNA sequence which encodes a protein or peptide or fragment or derivative thereof which includes an epitope which is recognized by cytotoxic T lymphocytes, said mycobacteria being administered in an amount effective to induce a CTL response in an animal.

2. The method of Claim 1 wherein said protein or peptide or fragment or derivative thereof includes an epitope which is recognized by cytotoxic T lymphocytes induced by an HIV protein or fragment or derivative thereof.

3. The method of Claim 2 wherein said protein or peptide or fragment or derivative thereof is an HIV protein or fragment or derivative thereof.

4. The method of Claim 1 wherein the mycobacteria are of the species M.bovis-BCG.

5. A composition for inducing a CTL response in an animal, comprising:

mycobacteria transformed with at least one DNA sequence which encodes a protein or peptide or fragment or derivative thereof which includes an epitope which is reocognized by cytotoxic T lymphocytes, and an acceptable pharmaceutical carrier, said mycobacteria being present in an amount effective to induce a CTL response in an animal.

6. The composition of Claim 5 wherein said protein or peptide or fragment or derivative thereof includes an epitope which is recognized by cytotoxic T lymphocytes induced by an HIV protein or fragment or derivative thereof.

7. The composition of Claim 6 wherein said protein or peptide or fragment or derivative thereof is an HIV protein or fragment or derivative thereof.

8. The composition of Claim 5 wherein said mycobacteria are of the species M.bovis-BCG.

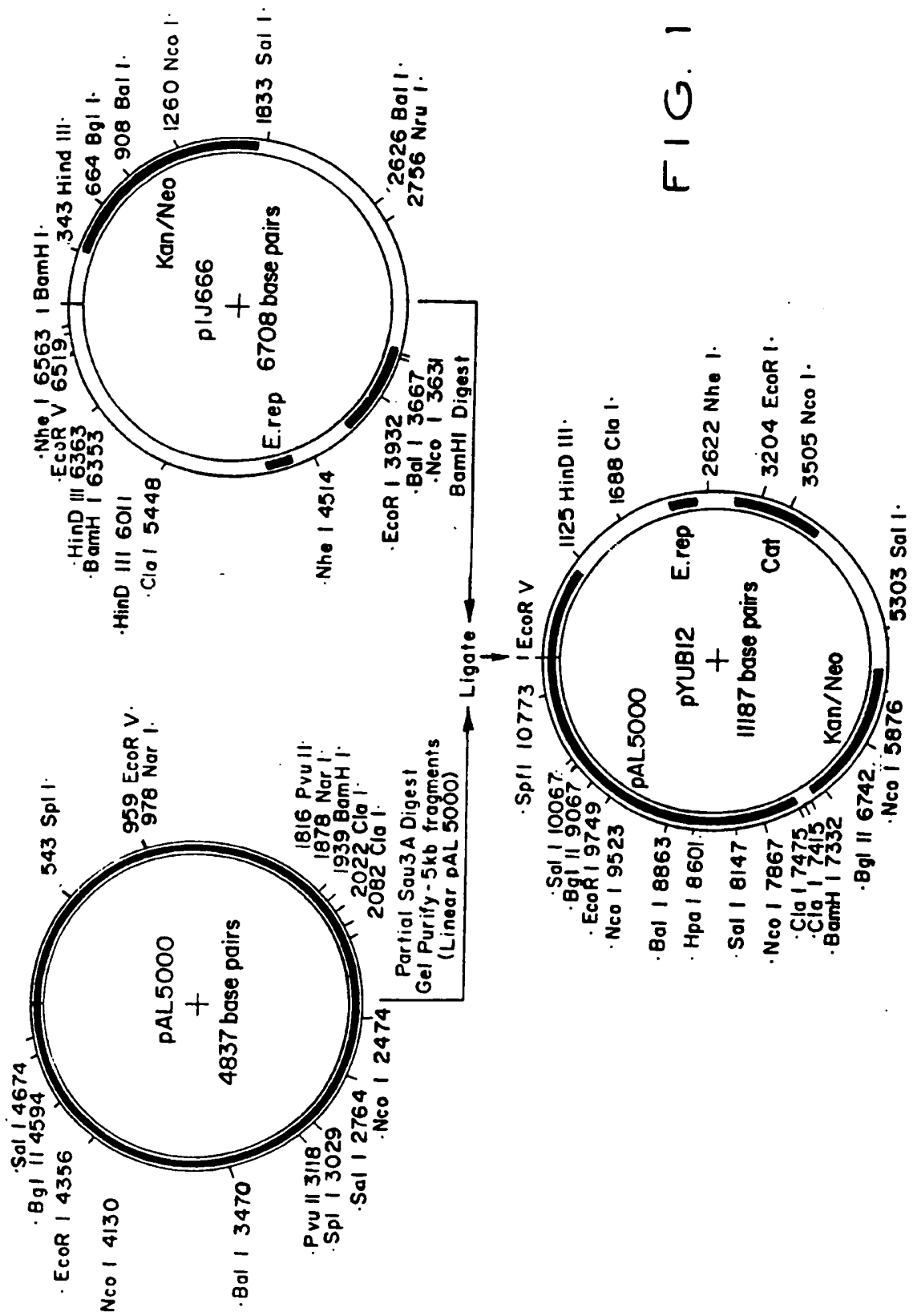
pIJ666
+
6708 base pairs
Kan/Neo
E.rep
Nhe I 6563
BamH I
343 Hind III
664 Bgl I
908 Bal I
1260 Nco I
1833 Sal I
2626 Bal I
2756 Nru I
EcoR V 6519
HinD III 6363
BamH I 6353
HinD III 6011
Cla I 5448
Nhe I 4514
EcoR I 3932
Bal I 3667
Nco I 3631
BamHI Digest
Ligate
pAL5000
+
4837 base pairs
543 Spl I
959 EcoR V
978 Nar I
1816 Pvu II
1878 Nar I
1939 BamH I
2022 Cla I
2082 Cla I
Partial Sau3A Digest
Gel Purify - 5kb fragments
(Linear pAL 5000)
Sal I 4674
Bgl II 4594
EcoR I 4356
Nco I 4130
Bal I 3470
Pvu II 3118
Spl I 3029
Sal I 2764
Nco I 2474
SpfI 10773
EcoR V
1125 HinD III
1688 Cla I
2622 Nhe I
3204 EcoR I
3505 Nco I
5303 Sal I
Nco I 5876
Bgl II 6742
BamH I 7332
Cla I 7415
Cla I 7475
Nco I 7867
Sal I 8147
Hpa I 8601
Bal I 8863
Sal I 10067
Bgl II 9067
EcoR I 9749
Nco I 9523
pAL5000
pYUB12
+
11187 base pairs
E.rep
Cat
Kan/Neo

FIG. 1

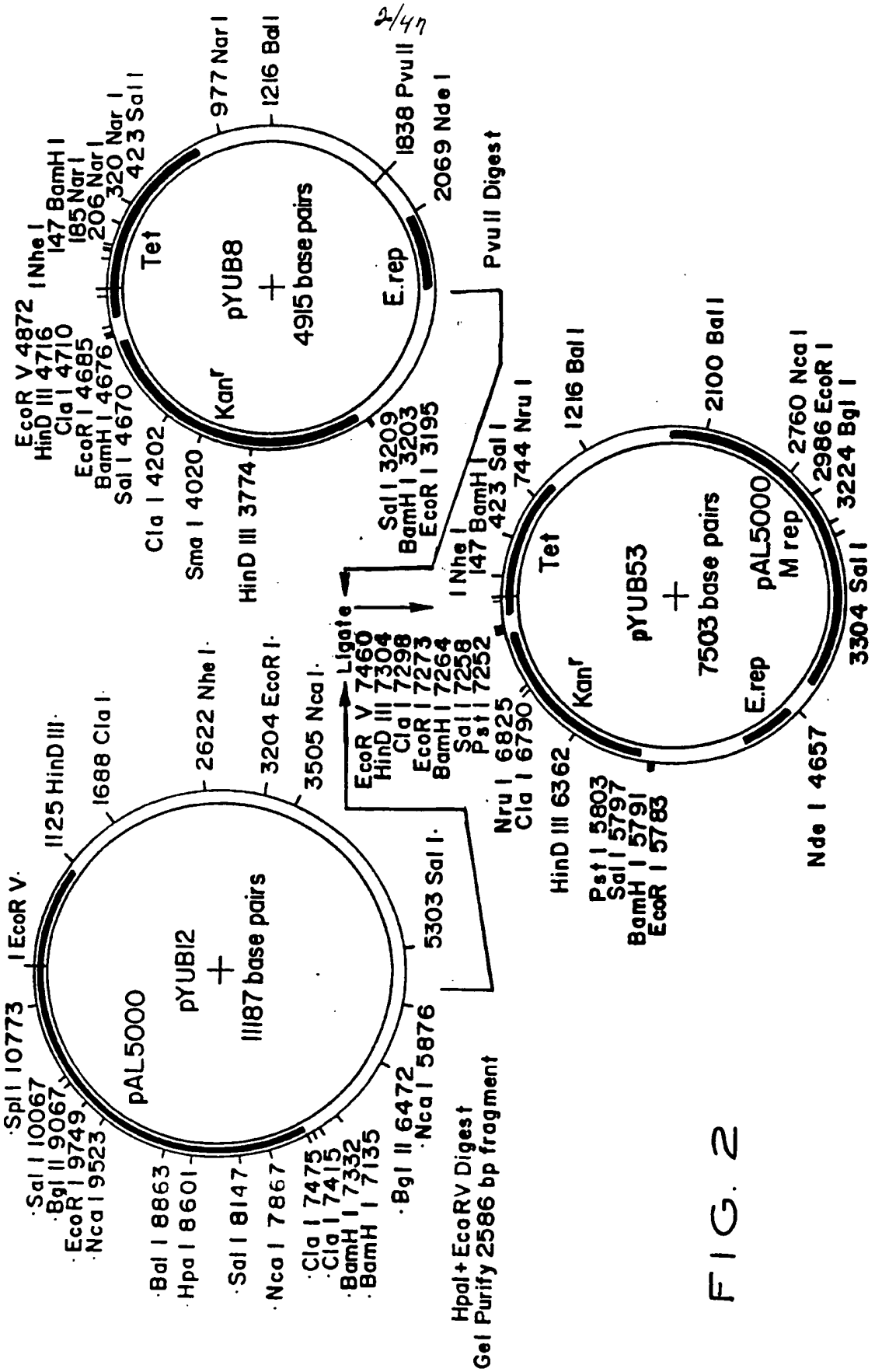
pYUB8
4915 base pairs
Tet
Kan^r
E.rep
EcoR V 4872
HinD III 4716
Cla I 4710
EcoR I 4685
BamH I 4676
Sal I 4670
Cla I 4202
Sma I 4020
HinD III 3774
Sal I 3209
BamH I 3203
EcoR I 3195
Nhe I
147 BamH I
185 Nar I
206 Nar I
320 Nar I
423 Sal I
977 Nar I
1216 Bal I
1838 Pvu II
2069 Nde I
Pvu II Digest
pYUB12
11187 base pairs
pAL5000
EcoR V
1125 HinD III
1688 Cla I
2622 Nhe I
3204 EcoR I
3505 Nca I
5303 Sal I
Spl I 10773
Sal I 10067
Bgl II 9067
EcoR I 9749
Nca I 9523
Bal I 8863
Hpa I 8601
Sal I 8147
Nca I 7867
Cla I 7475
Cla I 7415
BamH I 7332
BamH I 7135
Bgl II 6472
Nca I 5876
Hpa I + EcoRV Digest
Gel Purify 2586 bp fragment
Ligate
EcoR V 7460
HinD III 7304
Cla I 7298
EcoR I 7273
BamH I 7264
Sal I 7258
Pst I 7252
pYUB53
7503 base pairs
pAL5000
M rep
E.rep
Kan^r
Tet
Nhe I
147 BamH I
423 Sal I
744 Nru I
1216 Bal I
2100 Bal I
2760 Nca I
2986 EcoR I
3224 Bgl I
3304 Sal I
Nru I 6825
Cla I 6790
HinD III 6362
Pst I 5803
Sal I 5797
BamH I 5791
EcoR I 5783
Nde I 4657

FIG. 2

FIG. 3

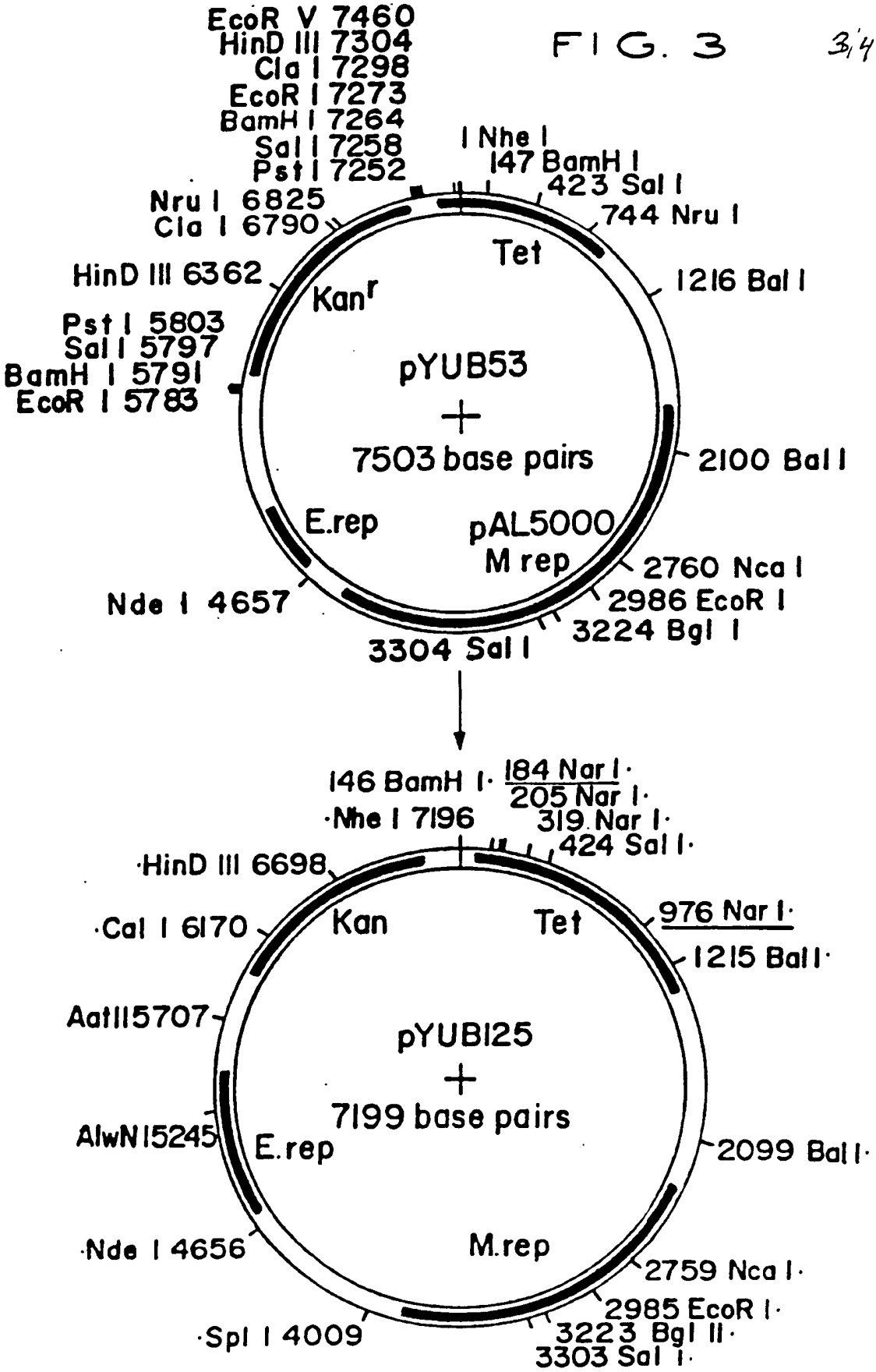
EcoR V 7460
HinD III 7304
Cla I 7298
EcoR I 7273
BamH I 7264
Sal I 7258
Pst I 7252
Nru I 6825
Cla I 6790
HinD III 6362
Pst I 5803
Sal I 5797
BamH I 5791
EcoR I 5783
I Nhe I
147 BamH I
423 Sal I
744 Nru I
Tet
1216 Bal I
Kan r
pYUB53
+
7503 base pairs
2100 Bal I
E.rep
pAL5000
M rep
2760 Nca I
2986 EcoR I
3224 Bgl I
Nde I 4657
3304 Sal I
146 BamH I
184 Nar I
205 Nar I
319 Nar I
Nhe I 7196
424 Sal I
HinD III 6698
Cal I 6170
Kan
Tet
976 Nar I
1215 Bal I
AatII 5707
pYUB125
+
7199 base pairs
AlwN I 5245
E.rep
2099 Bal I
Nde I 4656
M.rep
2759 Nca I
2985 EcoR I
Spl I 4009
3223 Bgl II
3303 Sal I

FIG. 4

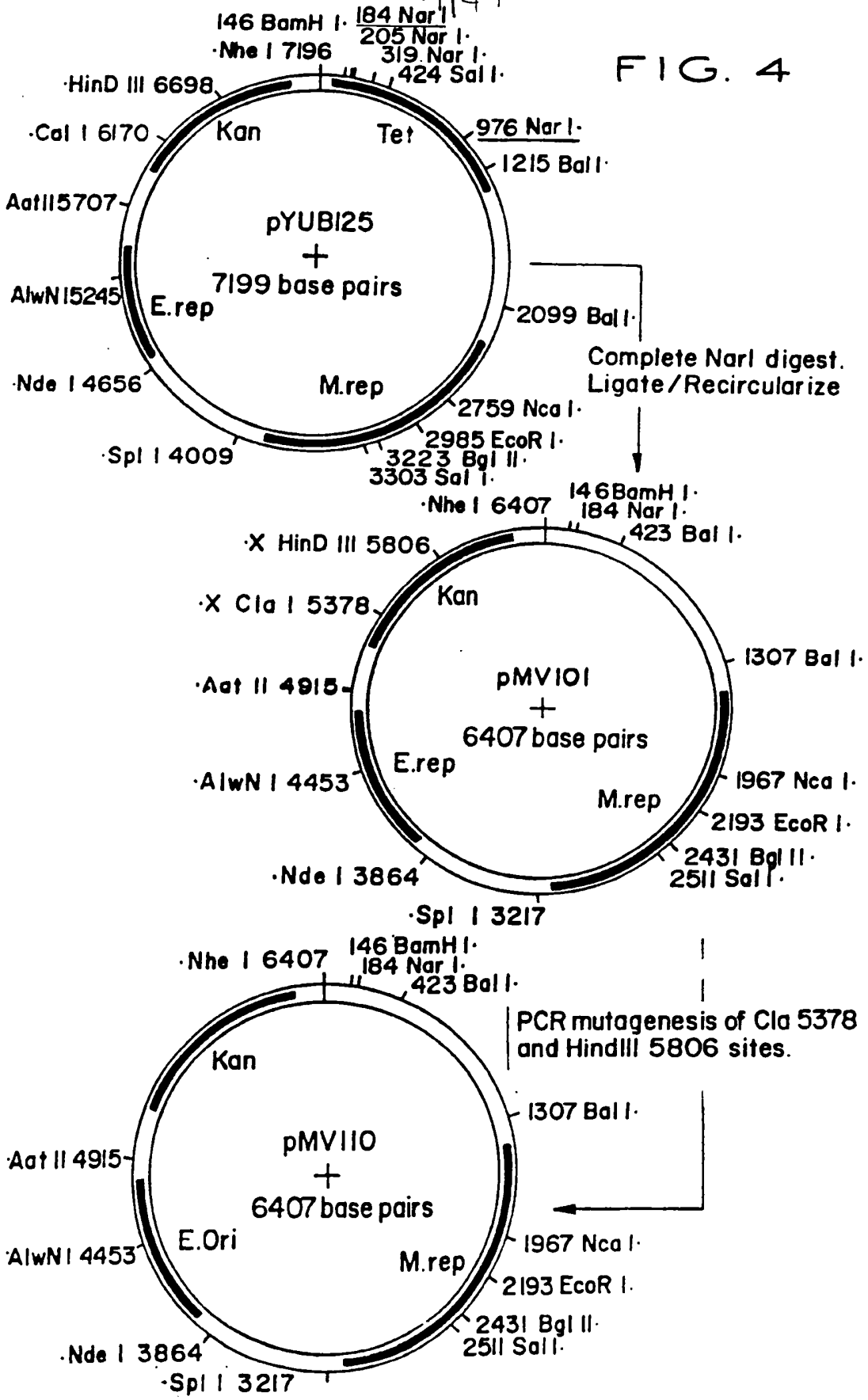
146 BamH I
184 Nar I
205 Nar I
319 Nar I
424 Sal I
Nhe I 7196
HinD III 6698
Cal I 6170
Kan
Tet
976 Nar I
1215 Ball
Aat II 5707
pYUB125
+
7199 base pairs
AlwN I 5245
E.rep
2099 Ball
Nde I 4656
M.rep
2759 Nca I
2985 EcoR I
Spl I 4009
3223 Bgl II
3303 Sal I
Complete NarI digest.
Ligate/Recircularize
Nhe I 6407
146 BamH I
184 Nar I
423 Bal I
X HinD III 5806
X Cla I 5378
Kan
1307 Bal I
Aat II 4915
pMV101
+
6407 base pairs
E.rep
AlwN I 4453
1967 Nca I
M.rep
2193 EcoR I
2431 Bgl II
2511 Sal I
Nde I 3864
Spl I 3217
Nhe I 6407
146 BamH I
184 Nar I
423 Bal I
PCR mutagenesis of Cla 5378
and HindIII 5806 sites.
Kan
1307 Bal I
Aat II 4915
pMV110
+
6407 base pairs
E.Ori
AlwN I 4453
1967 Nca I
M.rep
2193 EcoR I
2431 Bgl II
2511 Sal I
Nde I 3864
Spl I 3217

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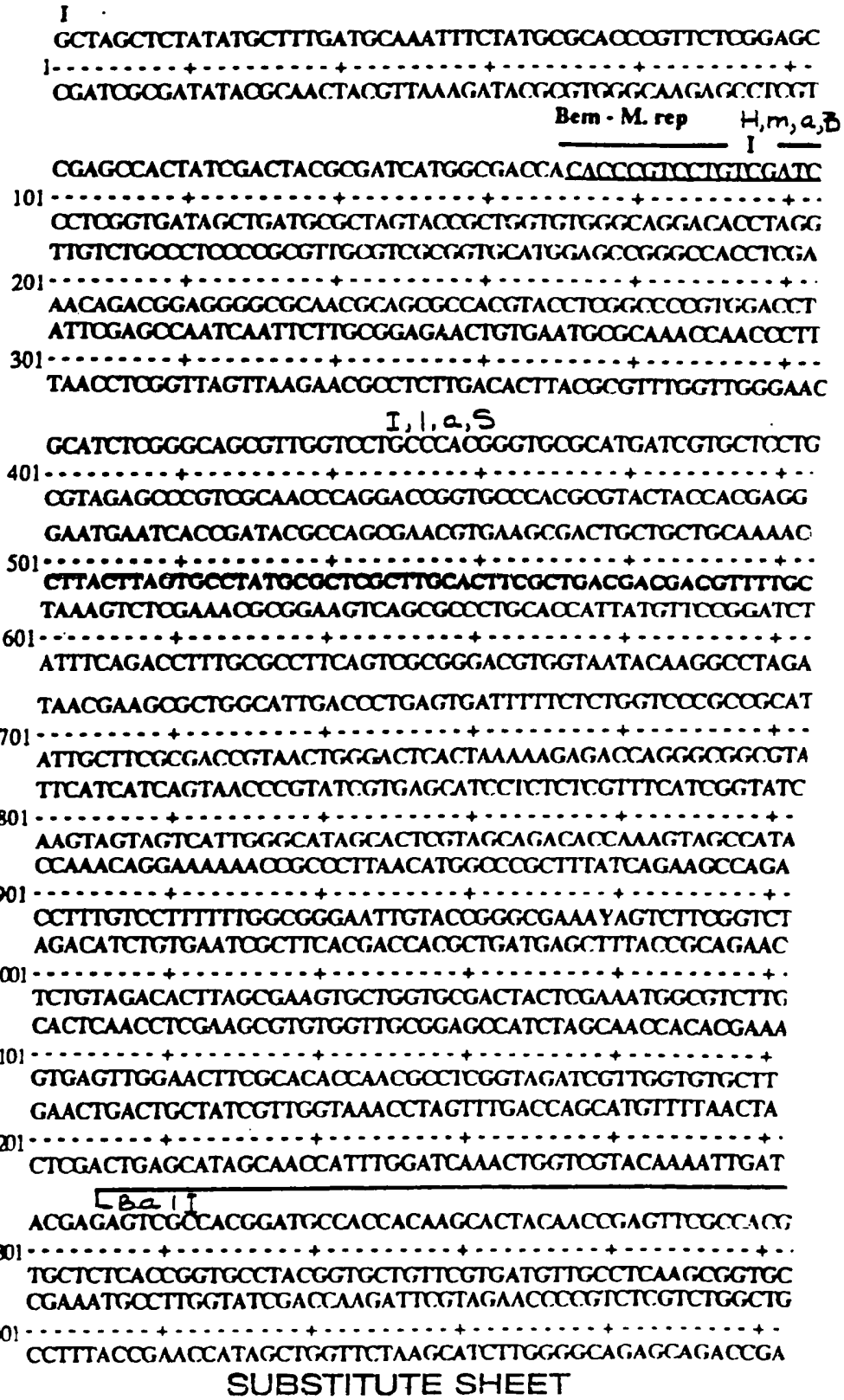
FIG. 5A



MATCH WITH FIG. 5B

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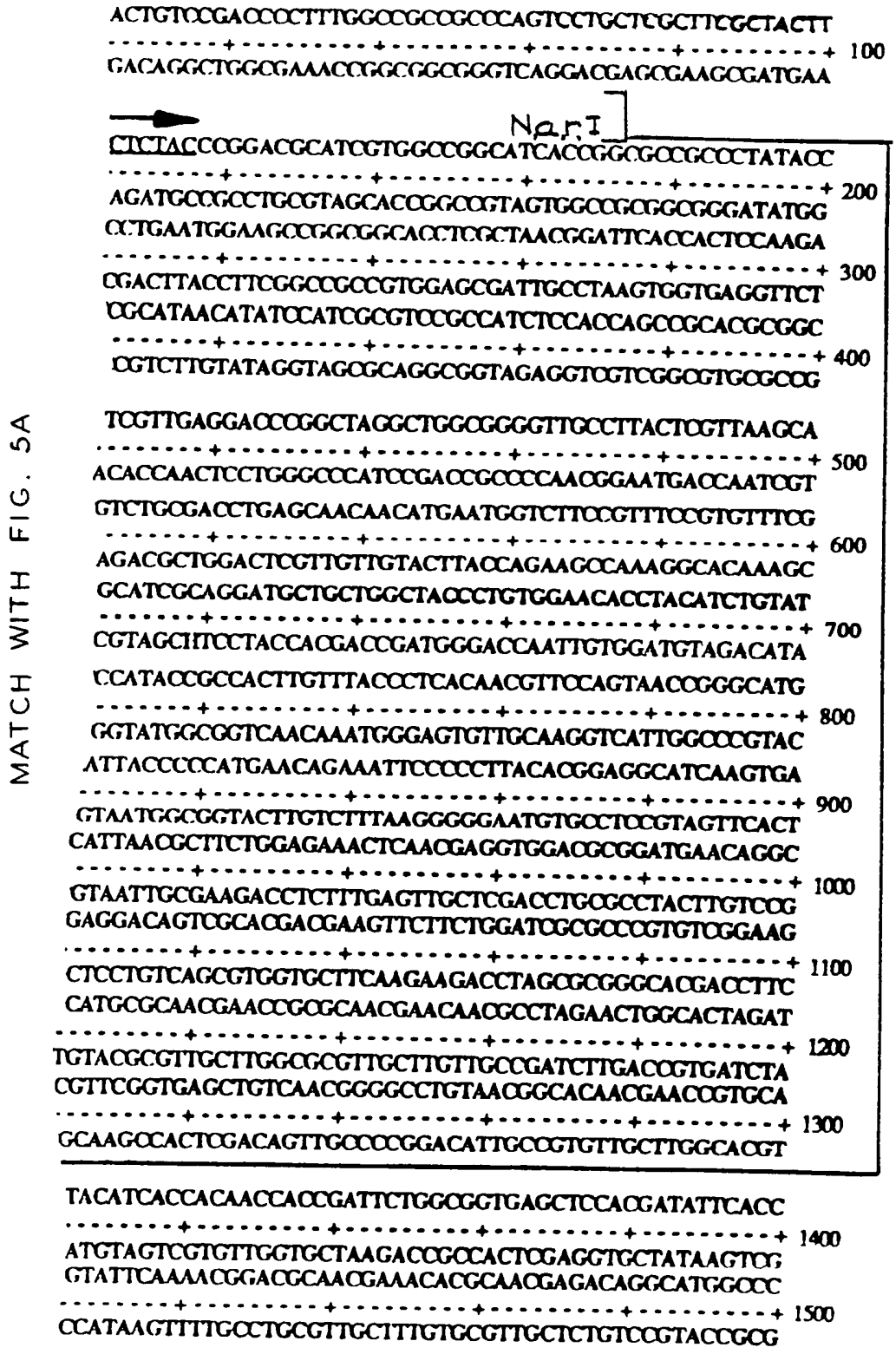
FIG. 5B



MATCH WITH FIG. 5A

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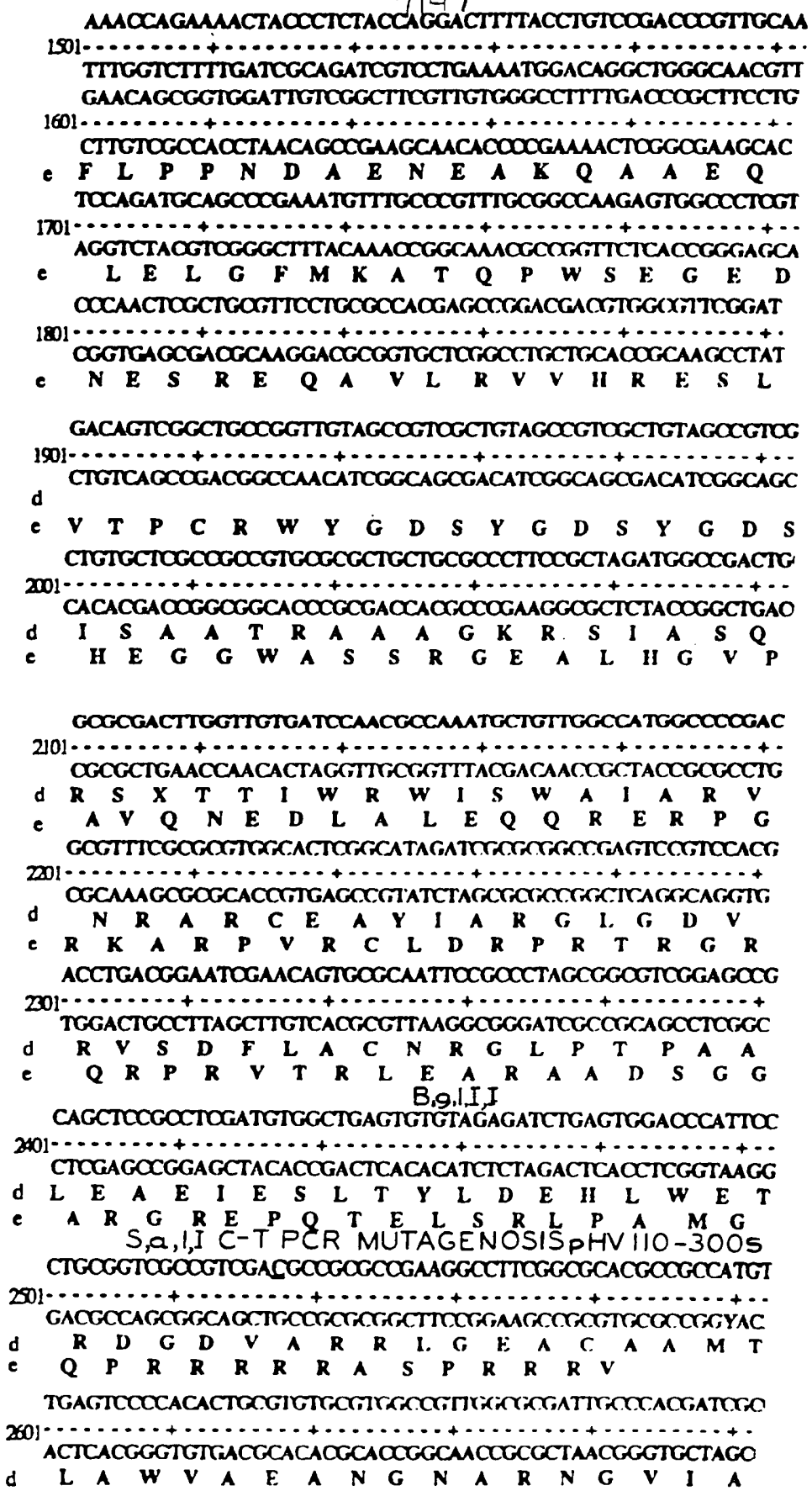

FIG. 5aA

MATCH WITH FIG. 5aB

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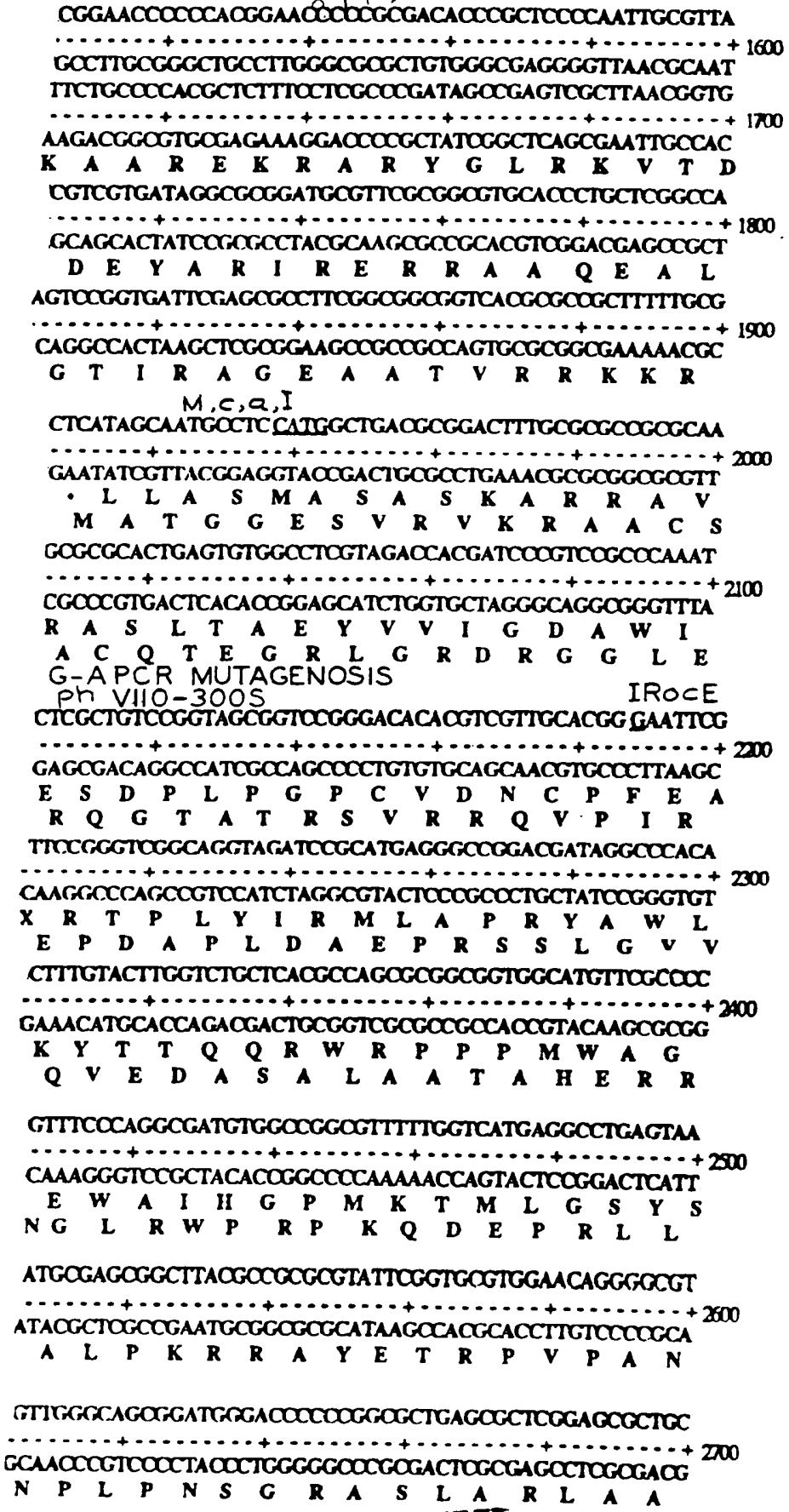


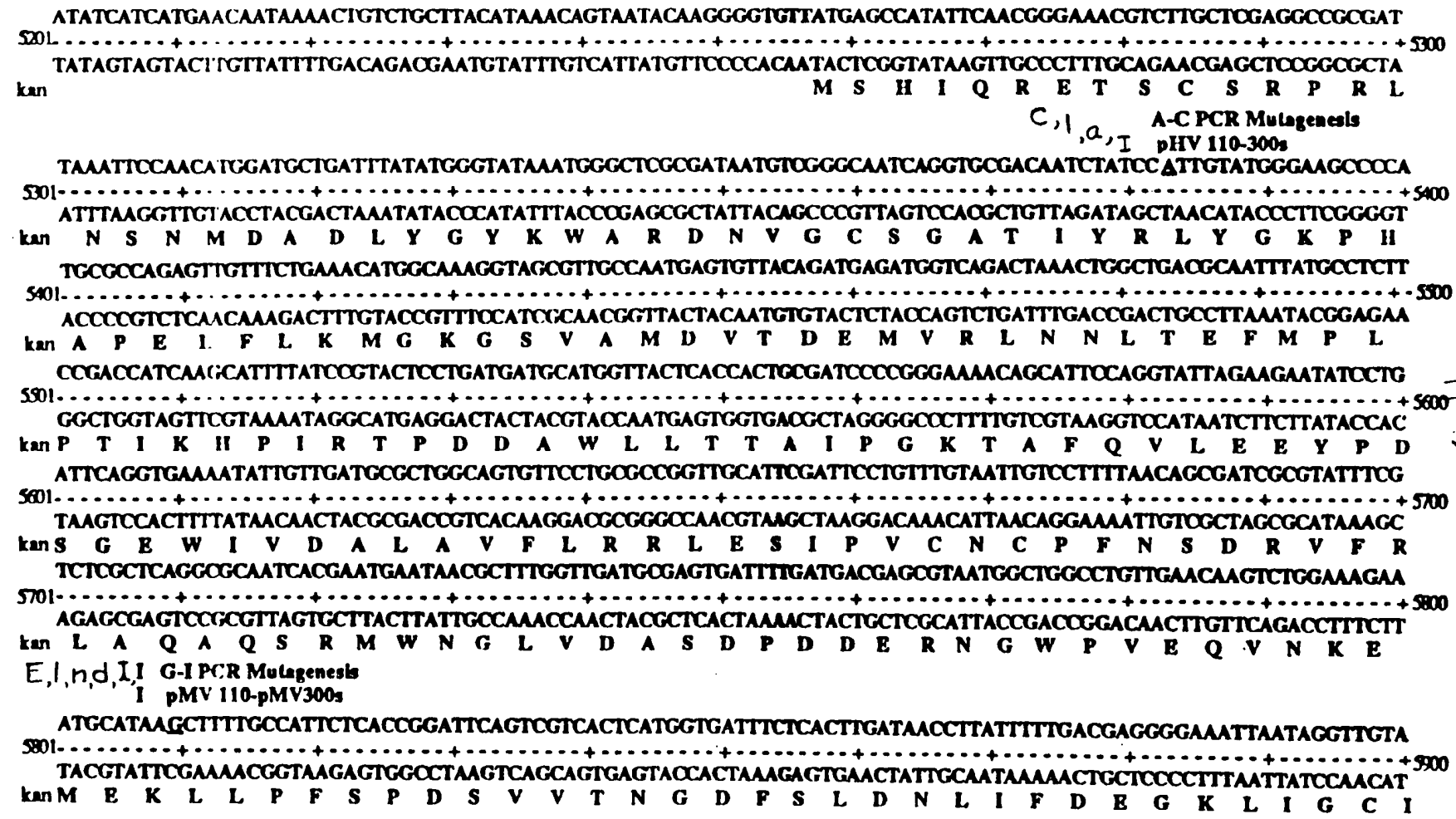
MATCH WITH FIG. 5aA

FIG. 5aB

SUBSTITUTE SHEET

FIG. 5cA

MATCH WITH FIG. 5cB



SUBSTITUTE SHEET

FIG. 5cB MATCH WITH FIG. 5cA

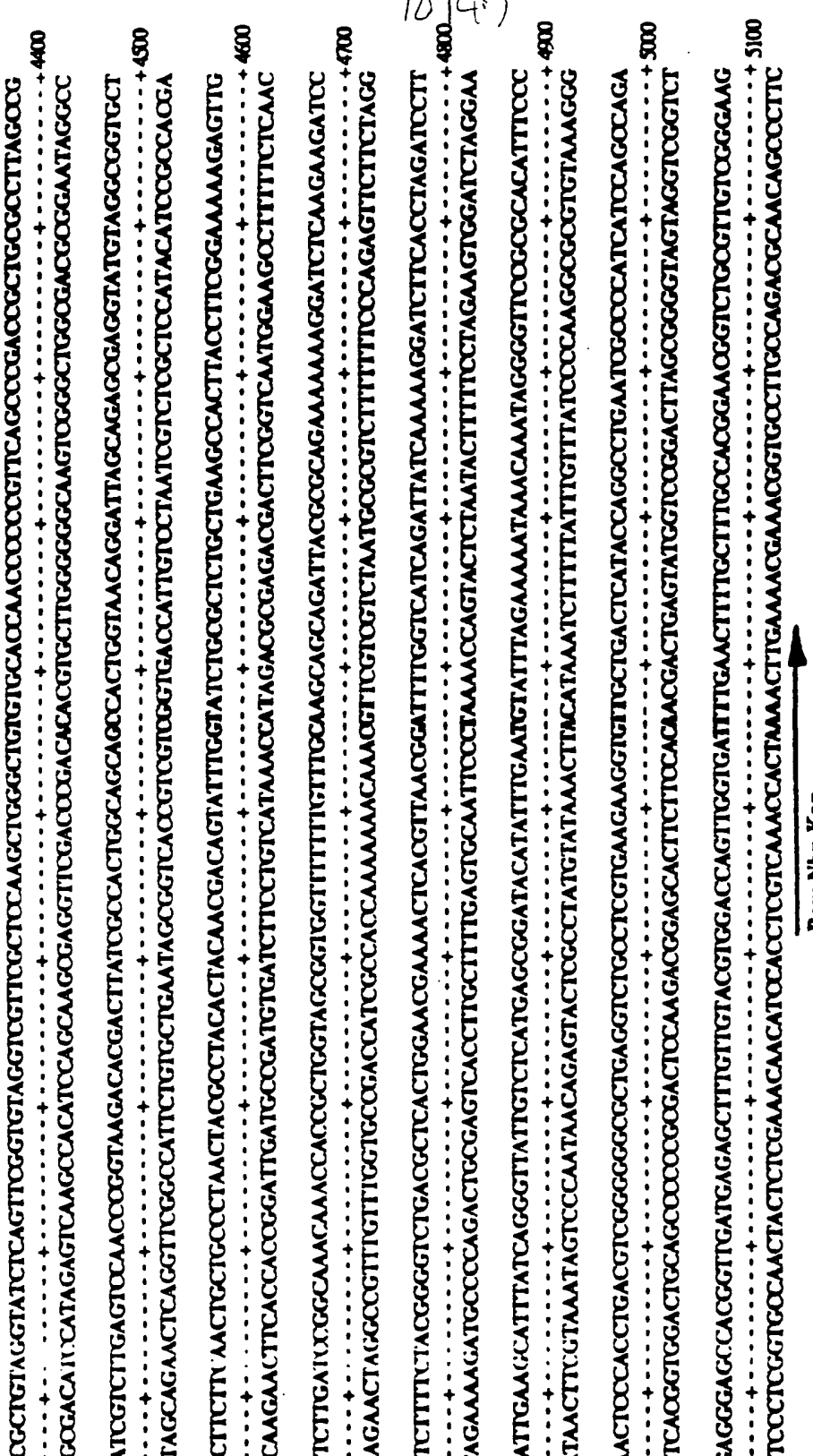
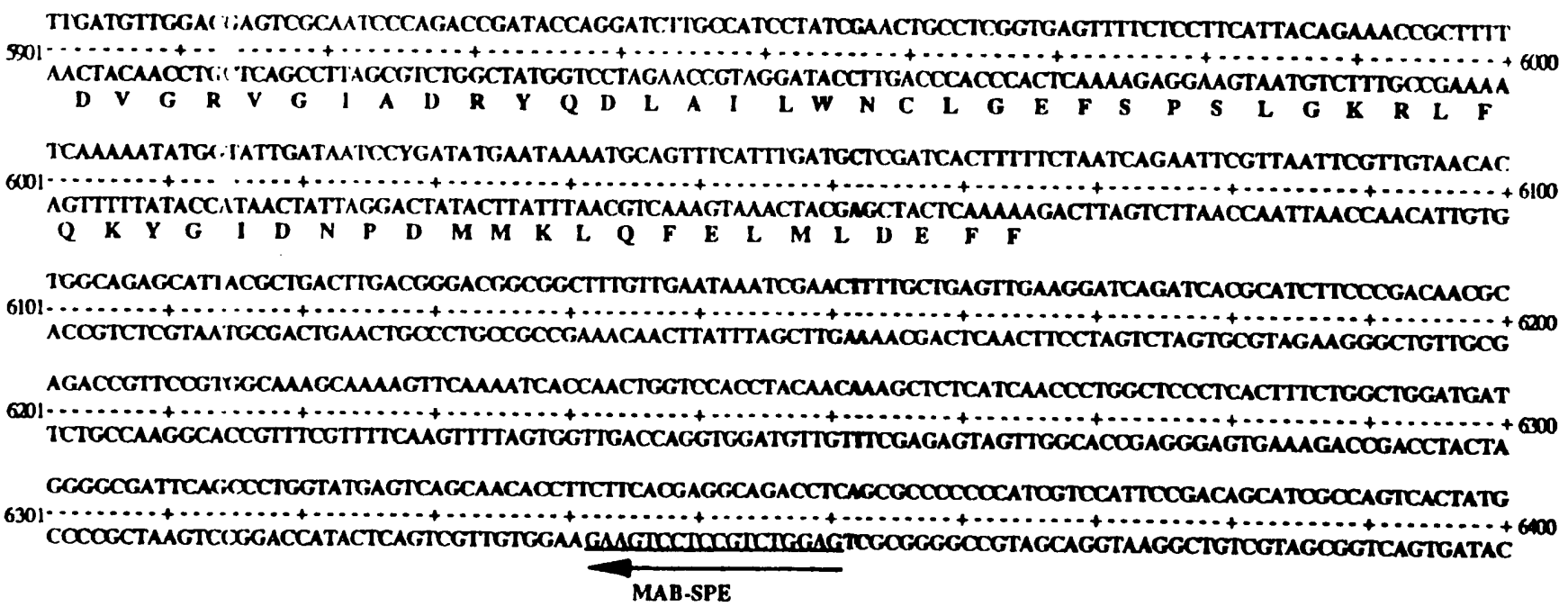


FIG. 5d



SUBSTITUTE SHEET

HSP 61 Cassette

```
   TCTAGAGGTGACCACAACGACGCGCCCGCTTTGATCGGGGACGTCTGCGGCCGACCATTTACGGGTCTTGTTGTCGTTGGCGGTCATGGGCCGAACATAC
1  ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+100
   CGATCGCCACTGGTGTTGCTGCGCCGGGCGAAACTAGCCCCCTGCAGACGCCGGCTGGTAAATGCCCAGAACAACAG CAACCGCCAGTACCCGGCTTGTATG

   TCACCCGGATCCGAGGGCCGAAGGACAAGGTCGAACGAGGGGCATGACCCGGTGCGGGGCTTCTTGCACTCGGCATAGGCGAGTGCTAAGAATAACGTTGG
101---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+200
   AGTGGGCCTAGCCTCCCGGCTCCTGTTCCAGCTTGCTCCCCGTACTGGGCCAGGCCCCGAAGAACGTGAGCCGTATCCGCTCACGATTCTTATTGCAACC

   CACTCGCGACCGGTGAGTCGTAGGTCGGGACGGTGAGGCCAGGCCCGTCGTCGCAGCGAGTGGCAGCGAGGACAACTTGAGCCGTCCGTCGCGGGCACTG
201---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+300
   GTGAGCGCTGGCCACTCAGCATCCAGCCCTGCCACTCCGGTCCGGGCAGCAGGTCGCTCACCGTCGCTCCTGTTGAACTCGGCAGGCAGCGCCCGTGAC

   CGCCCGGCCAGCGTAAGTAGCGGGGTTGCCGTCACCCGGTGACCCCCGGTTTCATCCCCGATCCGGAGGAATCACTTCGCAATGGCCAAGACAATTGC GGATCC
301---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+404
   GCGGGCCGGTCGCATTCATCGCCCCCAACGGCAGTGGGCCACTGGGGGCCAAAGTAGGGGCTAGGCCTCCTTAGTGAAGCGTTACCGGTTCTGTTAACGCCTAGG
                                                                                                  M  A  K  T  I  A  D  P
```

FIG. 6

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FIG. 7

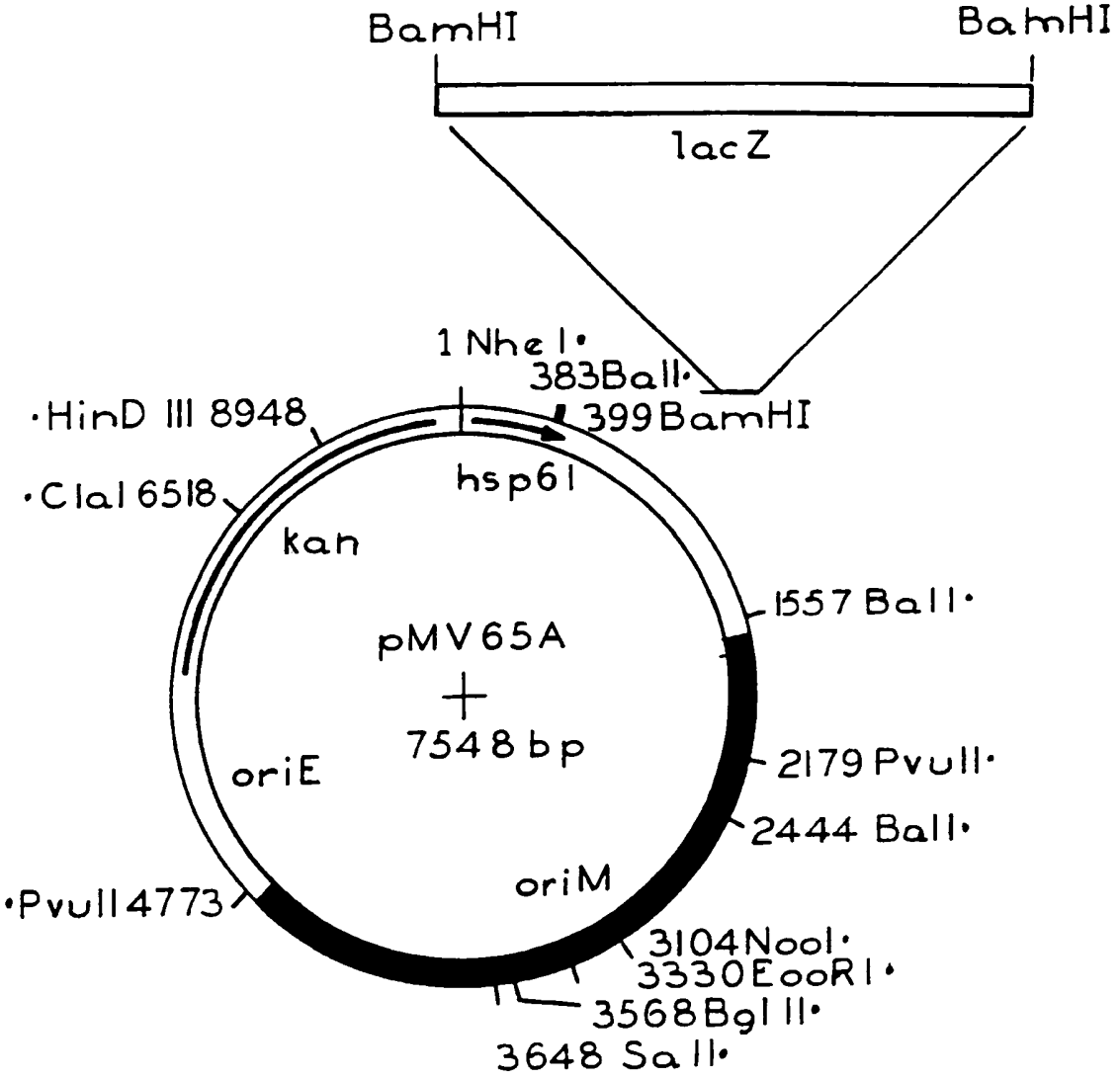
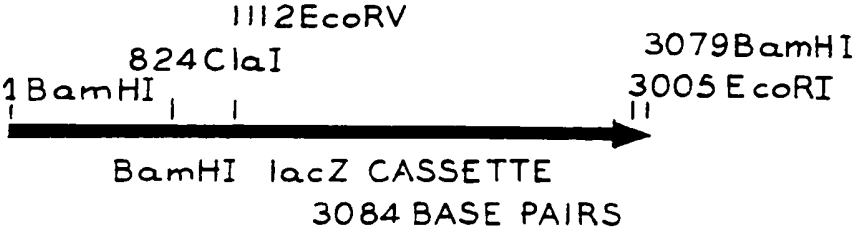


FIG. 8



14147

FIG. 9

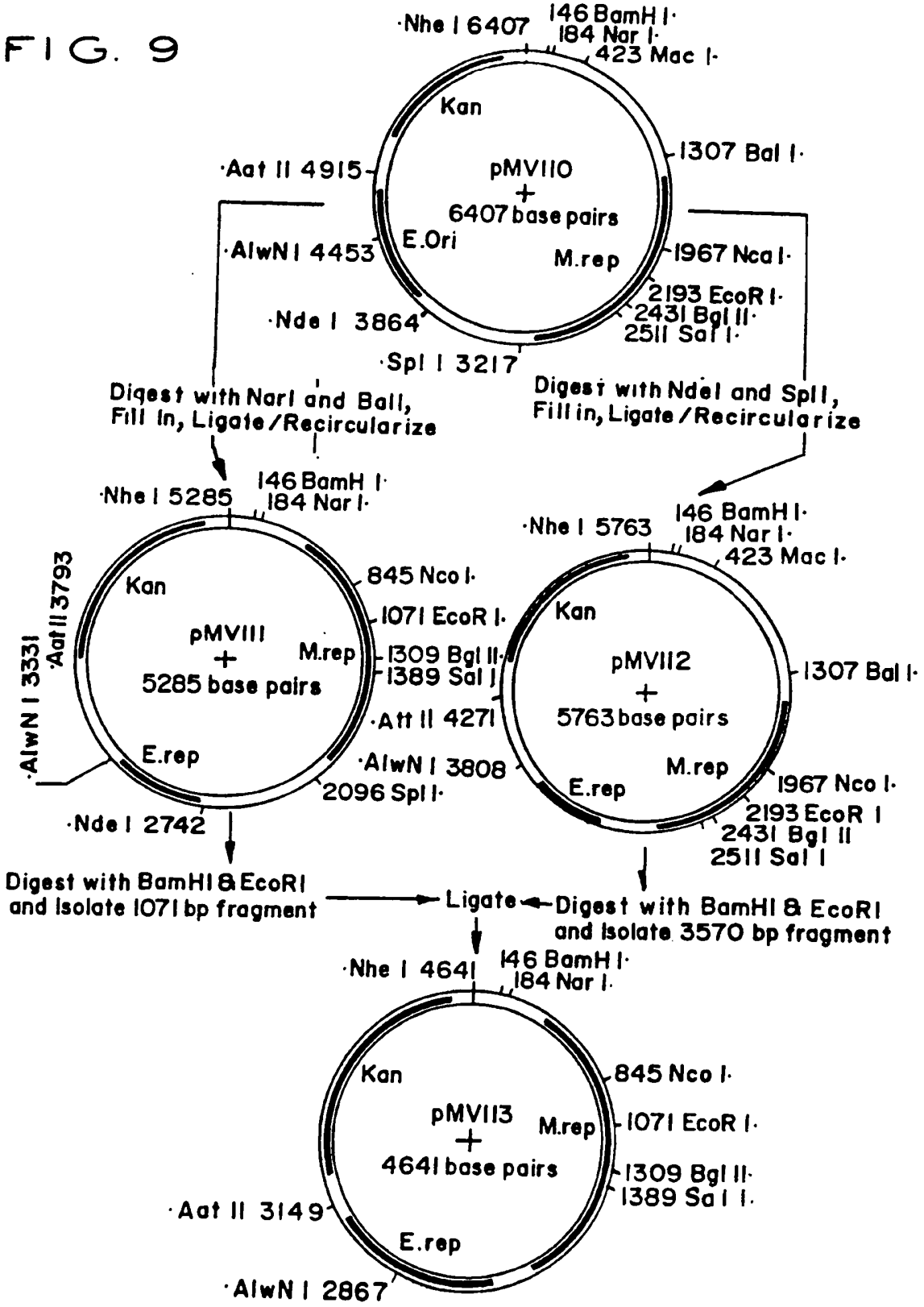
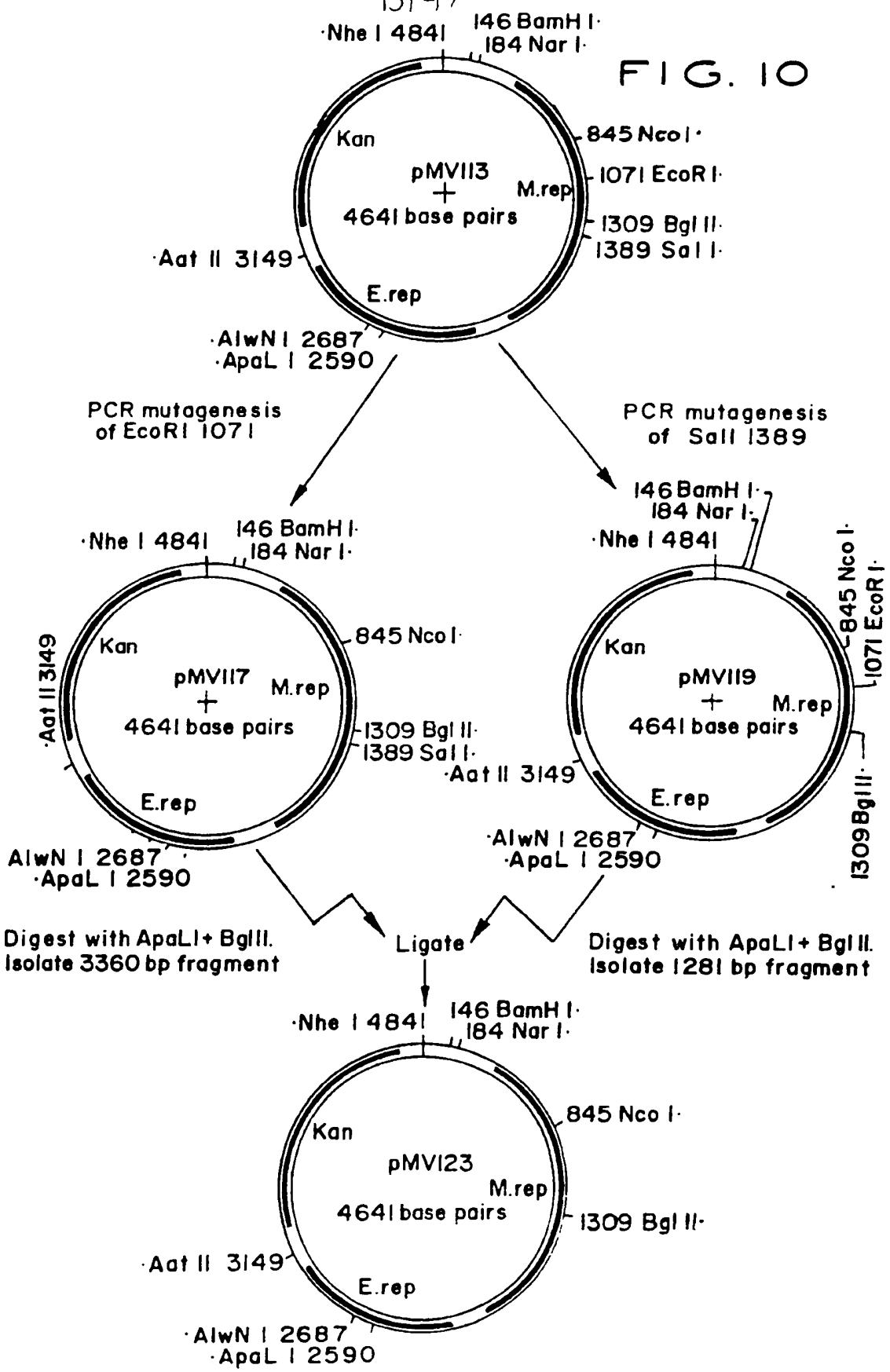

Nhe I 6407
146 BamH I
184 Nar I
423 Mac I
Kan
1307 Bal I
Aat II 4915
pMV110
+
6407 base pairs
AlwN I 4453
E.Ori
M.rep
1967 Nca I
2193 EcoR I
2431 Bgl II
2511 Sal I
Nde I 3864
Spl I 3217
Digest with NarI and BalI, Fill In, Ligate/Recircularize
Digest with NdeI and SplI, Fill in, Ligate/Recircularize
Nhe I 5285
146 BamH I
184 Nar I
Kan
845 Nco I
1071 EcoR I
Aat II 3793
AlwN I 3331
pMV111
+
5285 base pairs
M.rep
1309 Bgl II
1389 Sal I
Att II 4271
E.rep
AlwN I 3808
2096 Spl I
Nde I 2742
Nhe I 5763
146 BamH I
184 Nar I
423 Mac I
Kan
pMV112
+
5763 base pairs
1307 Bal I
M.rep
E.rep
1967 Nco I
2193 EcoR I
2431 Bgl II
2511 Sal I
Digest with BamHI & EcoRI and Isolate 1071 bp fragment
Ligate
Digest with BamHI & EcoRI and Isolate 3570 bp fragment
Nhe I 4641
146 BamH I
184 Nar I
Kan
845 Nco I
pMV113
+
4641 base pairs
M.rep
1071 EcoR I
1309 Bgl II
1389 Sal I
Aat II 3149
E.rep
AlwN I 2867

FIG. 10


Nhe I 4841
146 BamH I
184 Nar I
Kan
845 Nco I
pMV113
M.rep
1071 EcoR I
4641 base pairs
1309 Bgl II
1389 Sal I
Aat II 3149
E.rep
AlwN I 2687
ApaL I 2590
PCR mutagenesis of EcoR I 1071
PCR mutagenesis of Sal I 1389
pMV117
pMV119
Digest with ApaLI + BglII. Isolate 3360 bp fragment
Ligate
Digest with ApaLI + Bgl II. Isolate 1281 bp fragment
pMV123

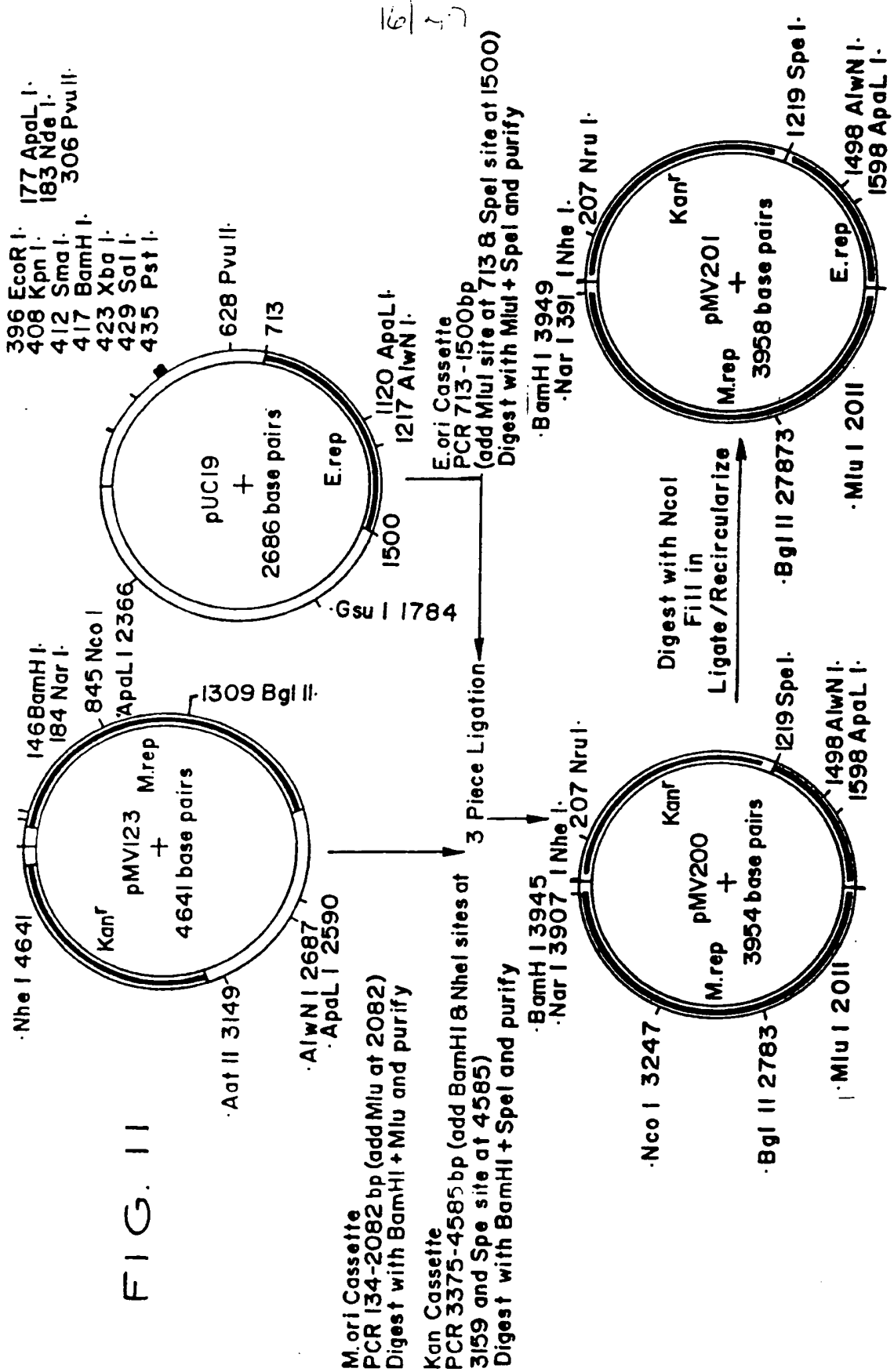

pMV123 + 4641 base pairs
Kan^r
M.rep
Nhe I 4641
146 BamH I
184 Nar I
845 Nco I
ApaL I 2366
1309 Bgl II
Aat II 3149
AlwN I 2687
ApaL I 2590
M.ori Cassette
PCR 134-2082 bp (add Mlu at 2082)
Digest with BamHI + Mlu and purify
pUC19 + 2686 base pairs
E.rep
396 EcoR I
408 Kpn I
412 Sma I
417 BamH I
423 Xba I
429 Sal I
435 Pst I
628 Pvu II
713
1120 ApaL I
1217 AlwN I
1500
Gsu I 1784
Kan Cassette
PCR 3375-4585 bp (add BamHI & NheI sites at 3159 and Spe site at 4585)
Digest with BamHI + SpeI and purify
3 Piece Ligation
pMV200 + 3954 base pairs
Kan^r
M.rep
Nco I 3247
Bgl II 2783
BamH I 3945
Nar I 3907
Nhe I
207 Nru I
1219 SpeI
1498 AlwN I
1598 ApaL I
Mlu I 2011
Digest with NcoI
Fill in
Ligate/Recircularize
pMV201 + 3958 base pairs
Kan^r
M.rep
E.rep
BamH I 3949
Nar I 391
Nhe I
207 Nru I
1219 Spe I
1498 AlwN I
1598 ApaL I
Bgl II 27873
Mlu I 2011
E.ori Cassette
PCR 713-1500bp
(add MluI site at 713 & SpeI site at 1500)
Digest with MluI + SpeI and purify

FIG. 11

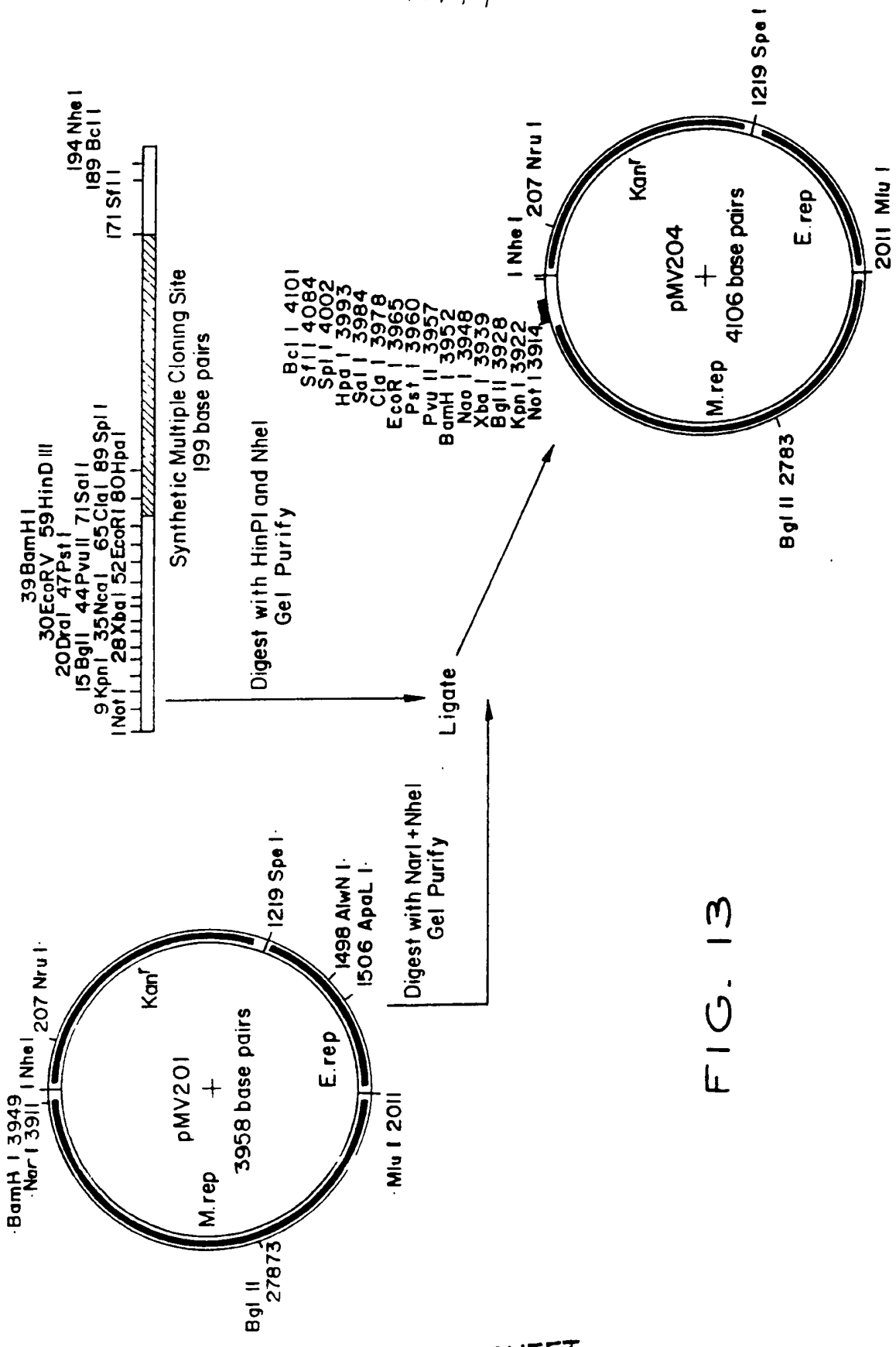
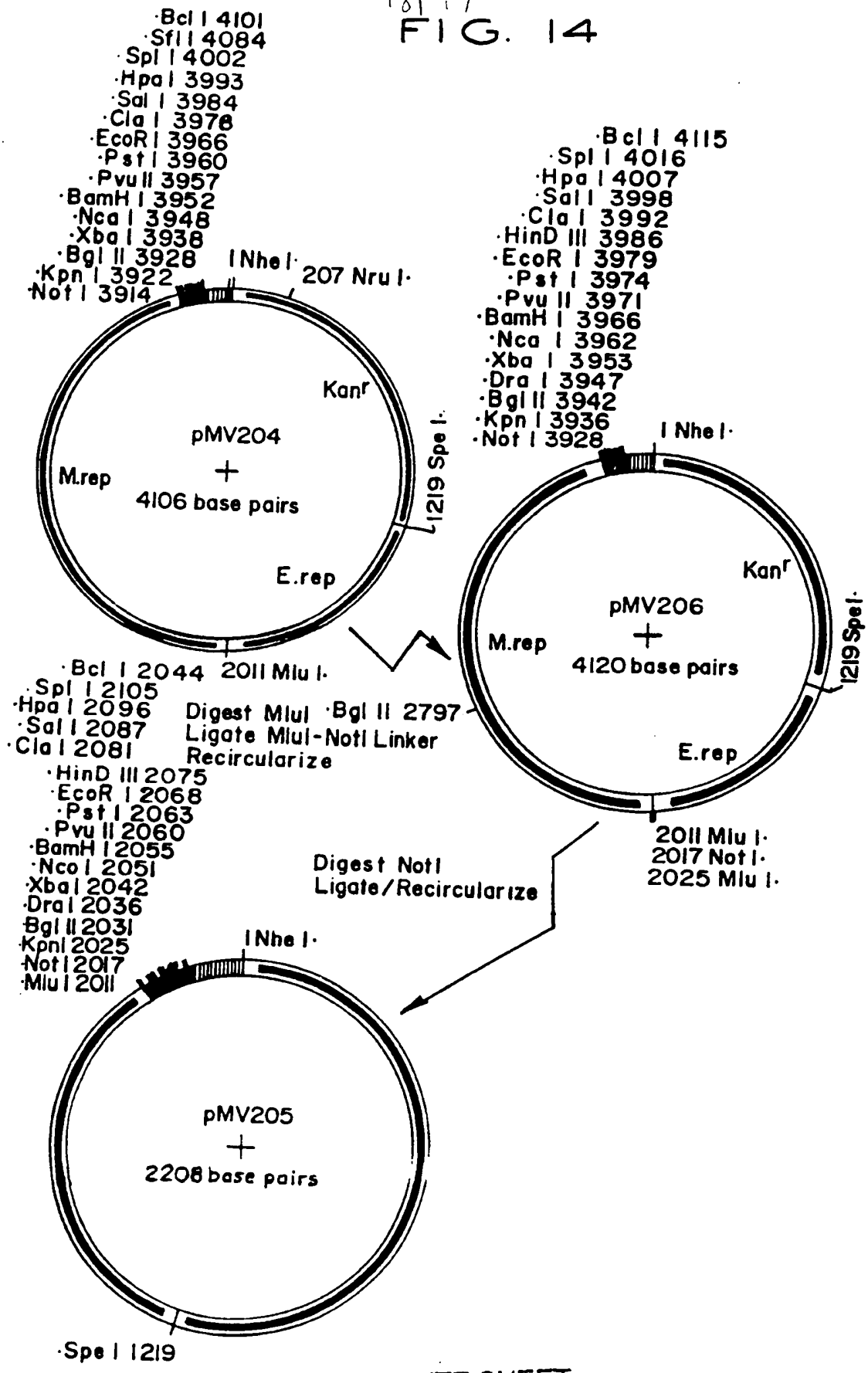

pMV201
+
3958 base pairs
Kan^r
M.rep
E.rep
BamH I 3949
Nar I 3911
I Nhe I
207 Nru I
1219 Spe I
1498 AlwN I
1506 ApaL I
Mlu I 2011
Bgl II 27873
Synthetic Multiple Cloning Site
199 base pairs
194 Nhe I
189 Bcl I
171 Sfl I
39 BamH I
30 EcoRV
59 HinD III
20 Dral
47 Pst I
15 Bgl I
44 Pvu II
71 Sal I
9 Kpn I
35 Nca I
65 Cla I
89 Spl I
1 Not I
28 Xba I
52 EcoR I
80 Hpa I
Digest with HinPI and NheI
Gel Purify
Bcl I 4101
Sfl I 4084
Spl I 4002
Hpa I 3993
Sal I 3984
Cla I 3978
EcoR I 3965
Pst I 3960
Pvu II 3957
BamH I 3952
Nae I 3948
Xba I 3939
Bgl II 3928
Kpn I 3922
Not I 3914
Ligate
Digest with NarI + NheI
Gel Purify
pMV204
+
4106 base pairs
Kan^r
M.rep
E.rep
I Nhe I
207 Nru I
1219 Spe I
2011 Mlu I
Bgl II 2783

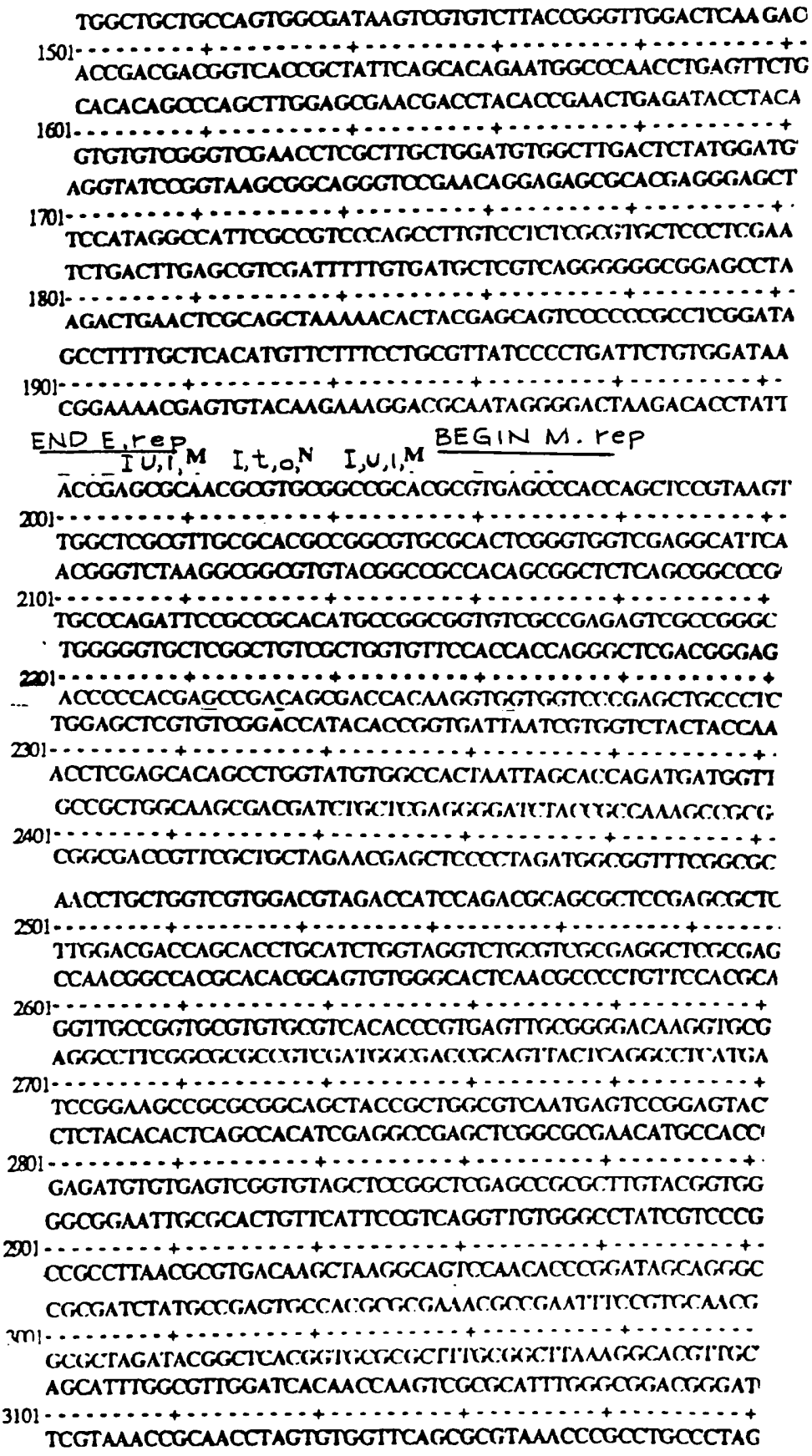
FIG. 13

18/47

FIG. 14

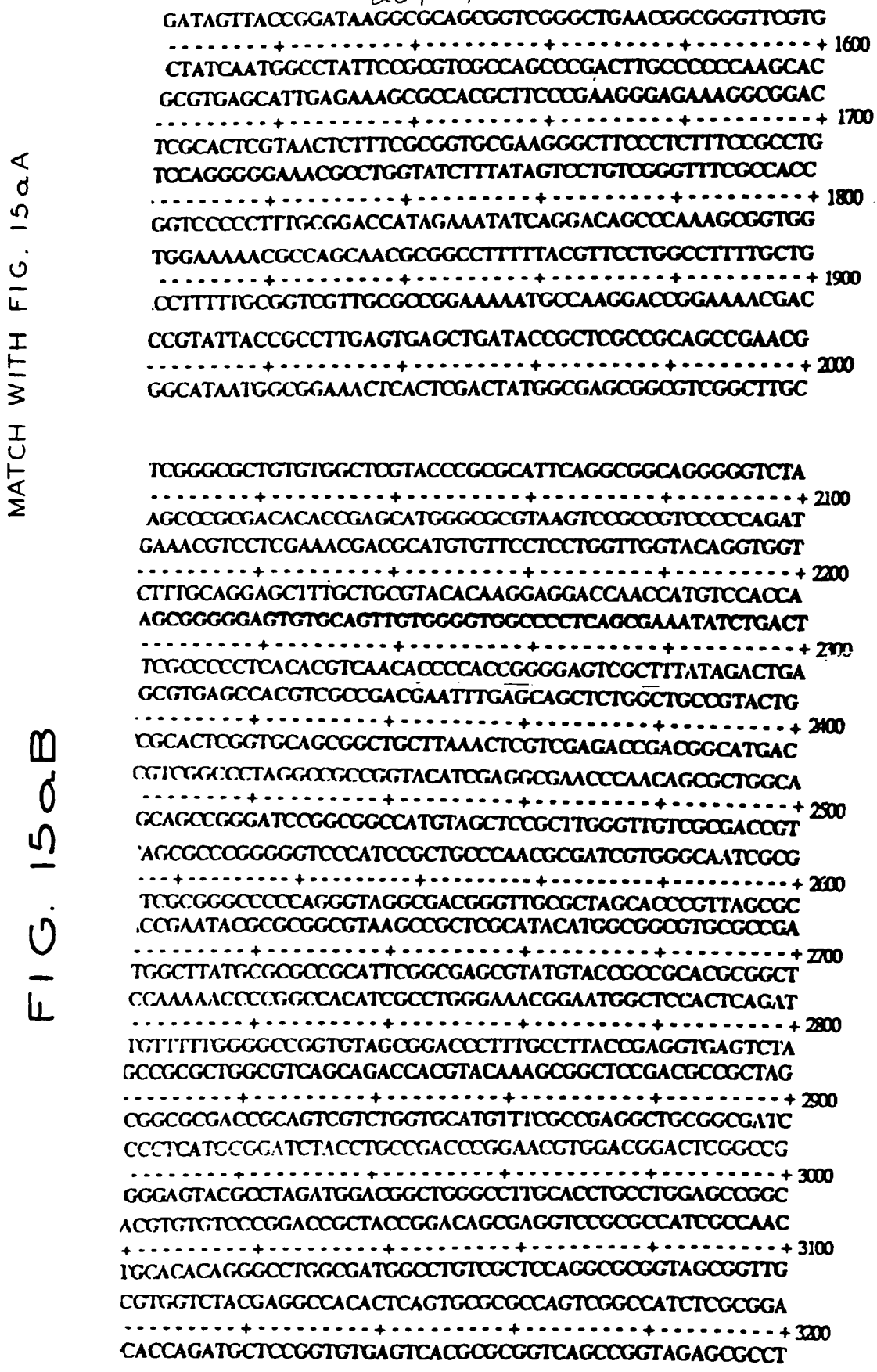


SUBSTITUTE SHEET



MATCH WITH FIG. 15aB

FIG. 15aA



MATCH WITH FIG. 15aA

FIG. 15aB

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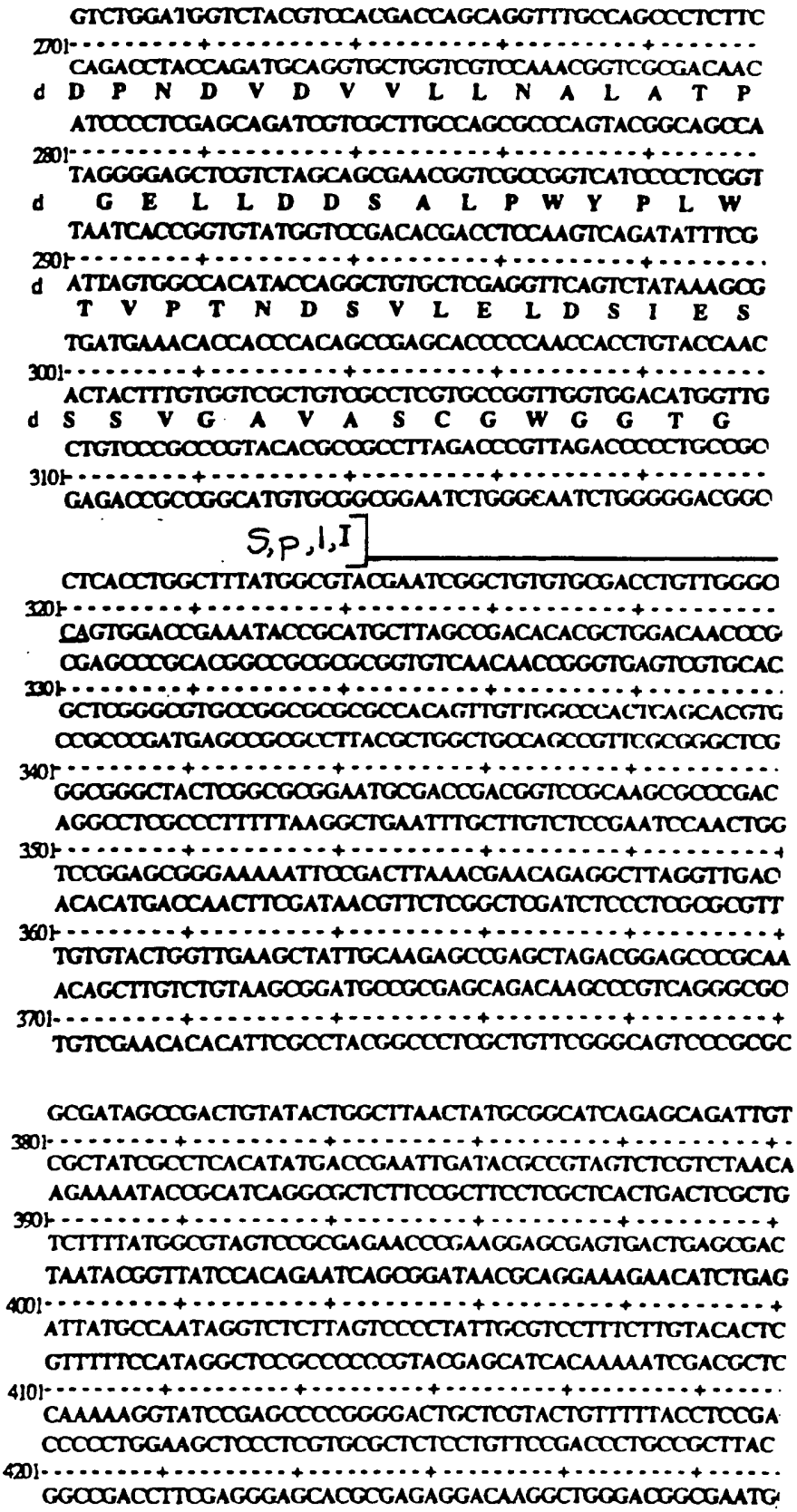
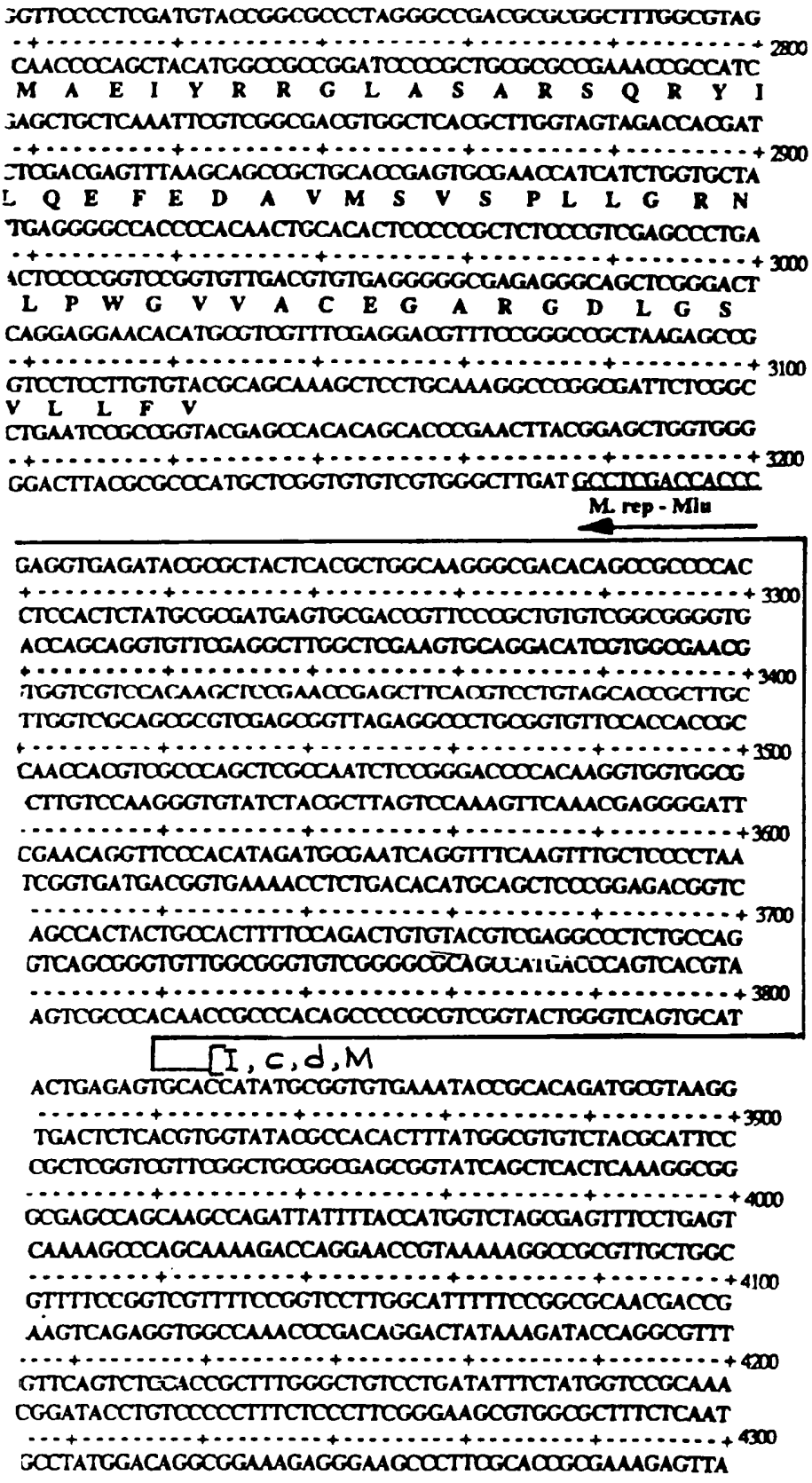


MATCH WITH FIG. 15bB

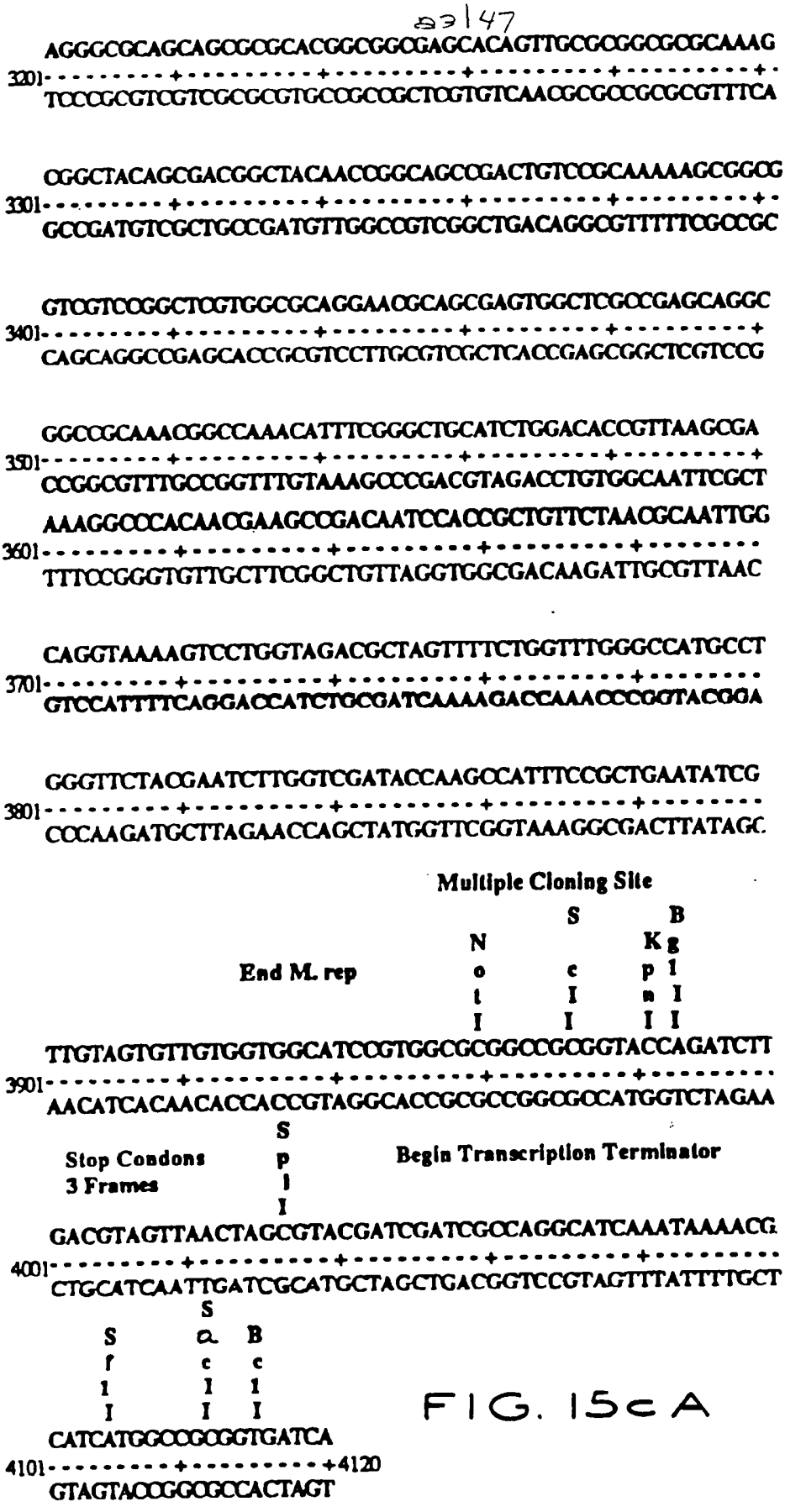
FIG. 15 bA

28147



DELETED IN MUI2-200S

FIG. 15B MATCH WITH FIG. 15A



MATCH WITH FIG. 15c B

FIG. 15c A

SUBSTITUTE SHEET

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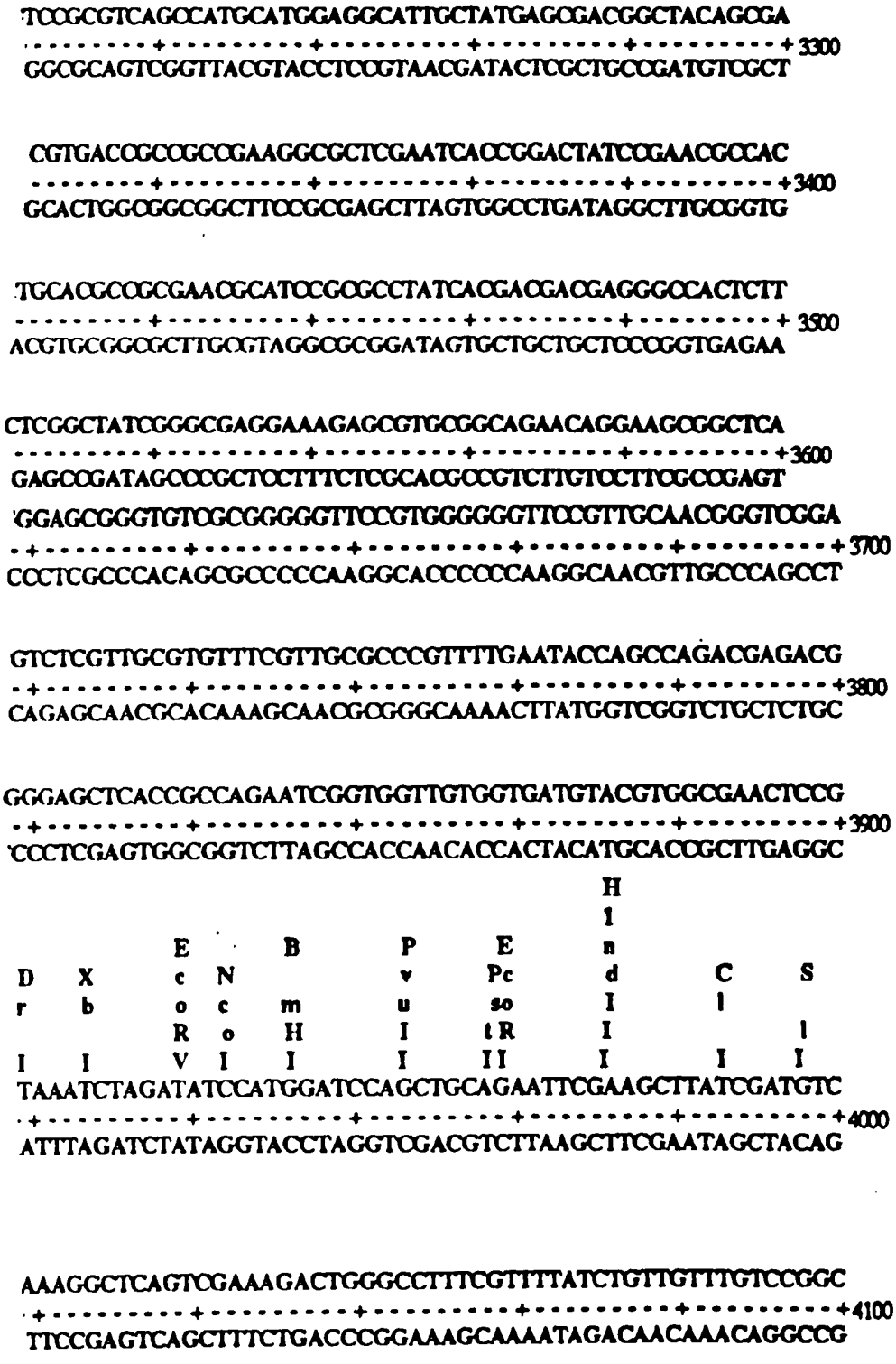
FIG. 15cB



MATCH WITH FIG. 15cA

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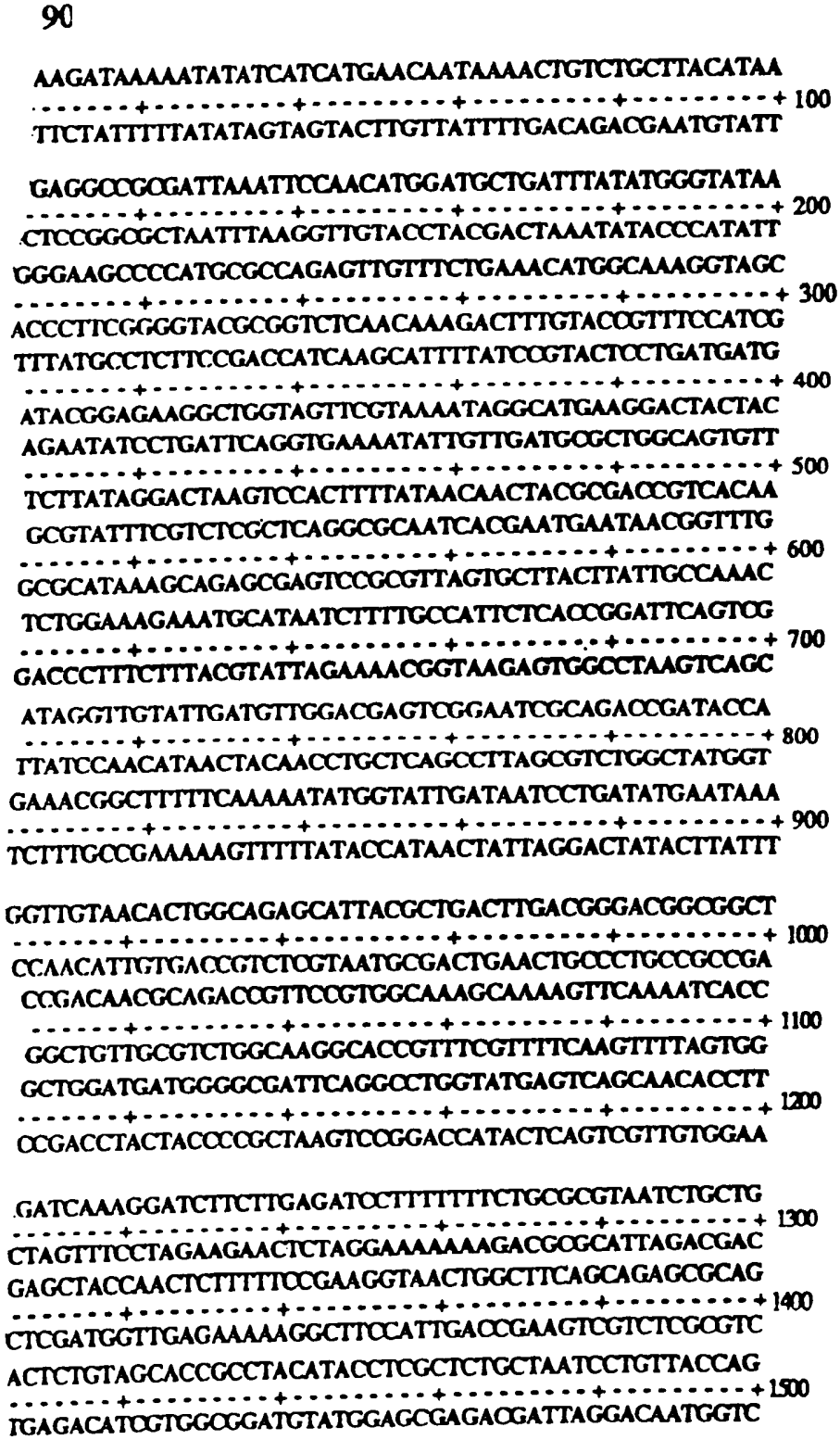
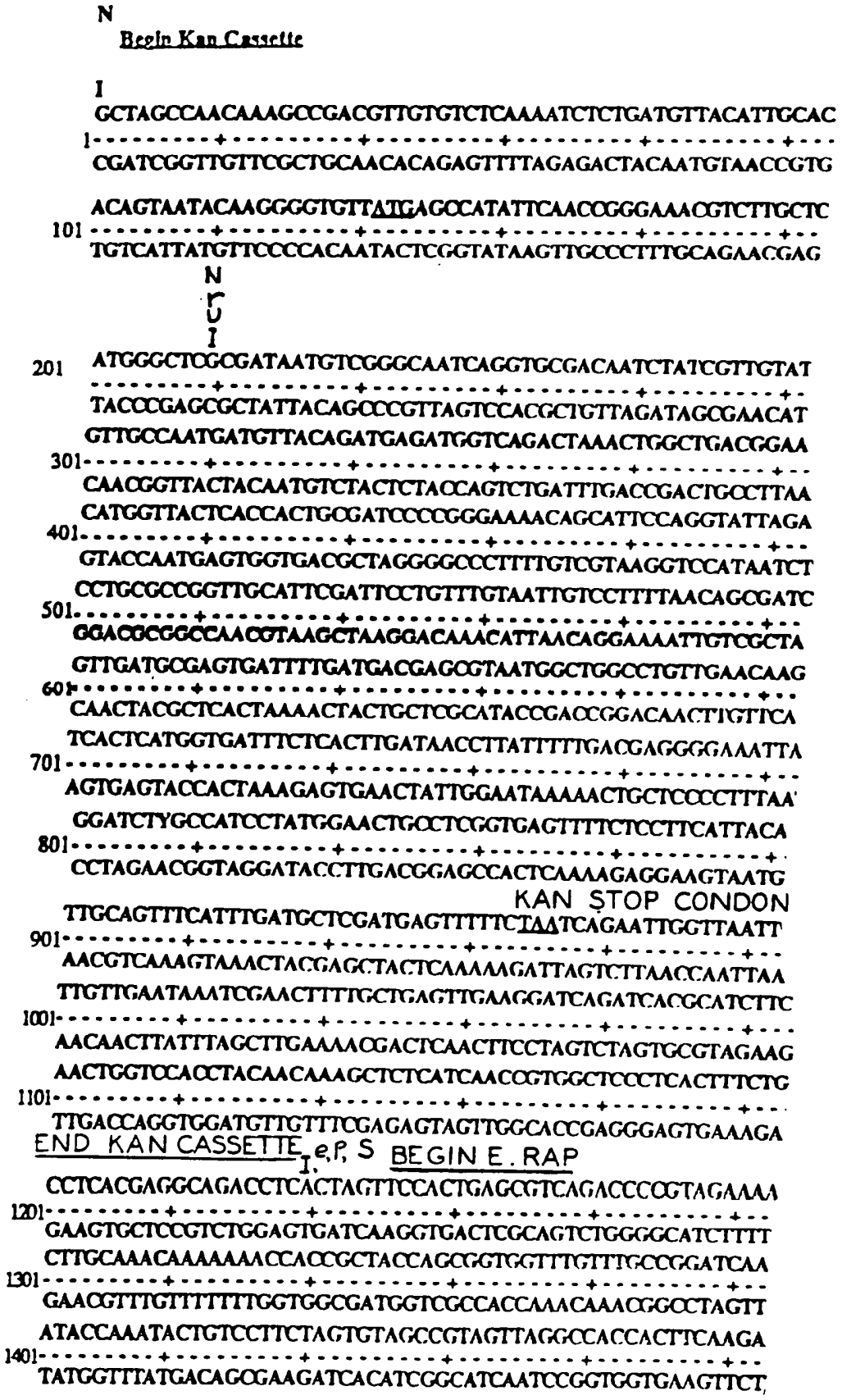
FIG. 15B



MATCH WITH FIG. 15A

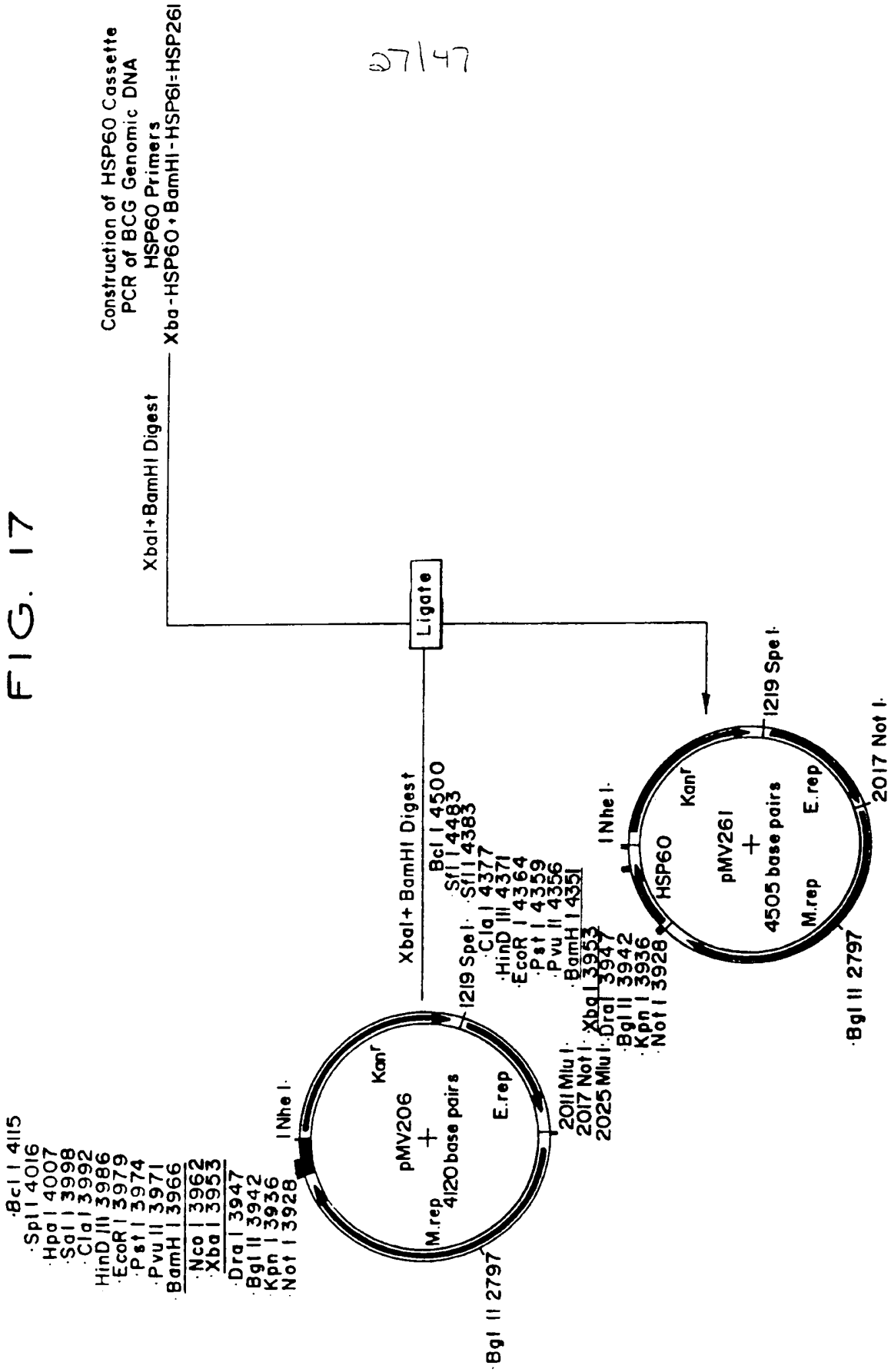
26 KH7

FIG. 15A



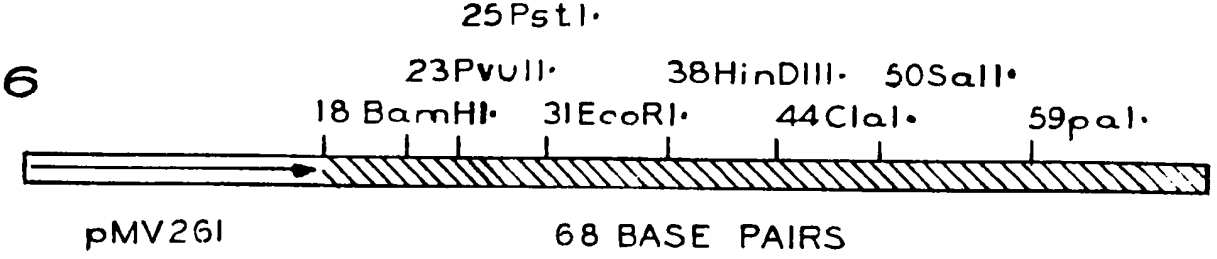
MATCH WITH FIG. 15B

FIG. 17


Construction of HSP60 Cassette
PCR of BCG Genomic DNA
HSP60 Primers
Xba-HSP60 • BamHI-HSP61-HSP261
XbaI+BamHI Digest
Ligate
XbaI+ BamHI Digest
pMV206
+
4120 base pairs
Kanr
E.rep
M.rep
NheI
1219 SpeI
2011 MluI
2017 NotI
2025 MluI
BglII 2797
pMV261
+
4505 base pairs
HSP60
Kanr
E.rep
M.rep
NheI
1219 SpeI
2017 NotI
BglII 2797
BclI 4115
SplI 4016
HpaI 4007
SalI 3998
ClaI 3992
HinDIII 3986
EcoRI 3979
PstI 3974
PvuII 3971
BamHI 3966
NcoI 3962
XbaI 3953
DraI 3947
BglII 3942
KpnI 3936
NotI 3928
BclI 4500
SfiI 4483
SfiI 4383
ClaI 4377
HinDIII 4371
EcoRI 4364
PstI 4359
PvuII 4356
BamHI 4351
XbaI 3953
DraI 3947
BglII 3942
KpnI 3936
NotI 3928

ATG GCC AAG ACA ATT GCG GAT CCA GCT GCA GAA TTC GAA GCT TAT CGA TGT CGA CGT AGT TAA CTA GC

FIG. 16



READING FRAMES AND RESTRICTION SITES IN MCS OF pMV261
THE HSP60 GENE SEQUENCE STARTS AT THE ATG START CODON OR BASE 4334 OF pMV261 AND ENDS AT THE UNDERLINED BamHI SITE. THE SYNTHETIC MCS BEGINS AT THE BamHI SITE. THE DIRECTION OF HSP60 TRANSCRIPTION AND TRANSLATION IS INDICATED BY ARROW. SYNTHETIC STOP CODON IN FRAME WITH THE START CODON OF HSP CASSETTE ARE ALSO UNDERLINED.

FIG. 12

SYNTHETIC MULTIPLE CLONING SITE (MCS) + STRAND

GAA GGC GCG GCC GCG GTA CCA GAT CTT TAA ATC TAG ATA TCC ATG GAT
CCA GCT GCA GAA TTC GAA GCT TAT CGA TGT CGA CGT AGT TAA CTA GCG
TAC GAT CGA CTG CCA GGC ATC AAA TAA AAC GAA AGG CTC AGT CGA AAG
ACT GGG CCT TTC GTT TTA TCT GTT GTT TGT CCG GCC ATC ATG GCC GCG
GTG ATC AGC TAG TAC G

SUBSTITUTE SHEET

FIG. 18

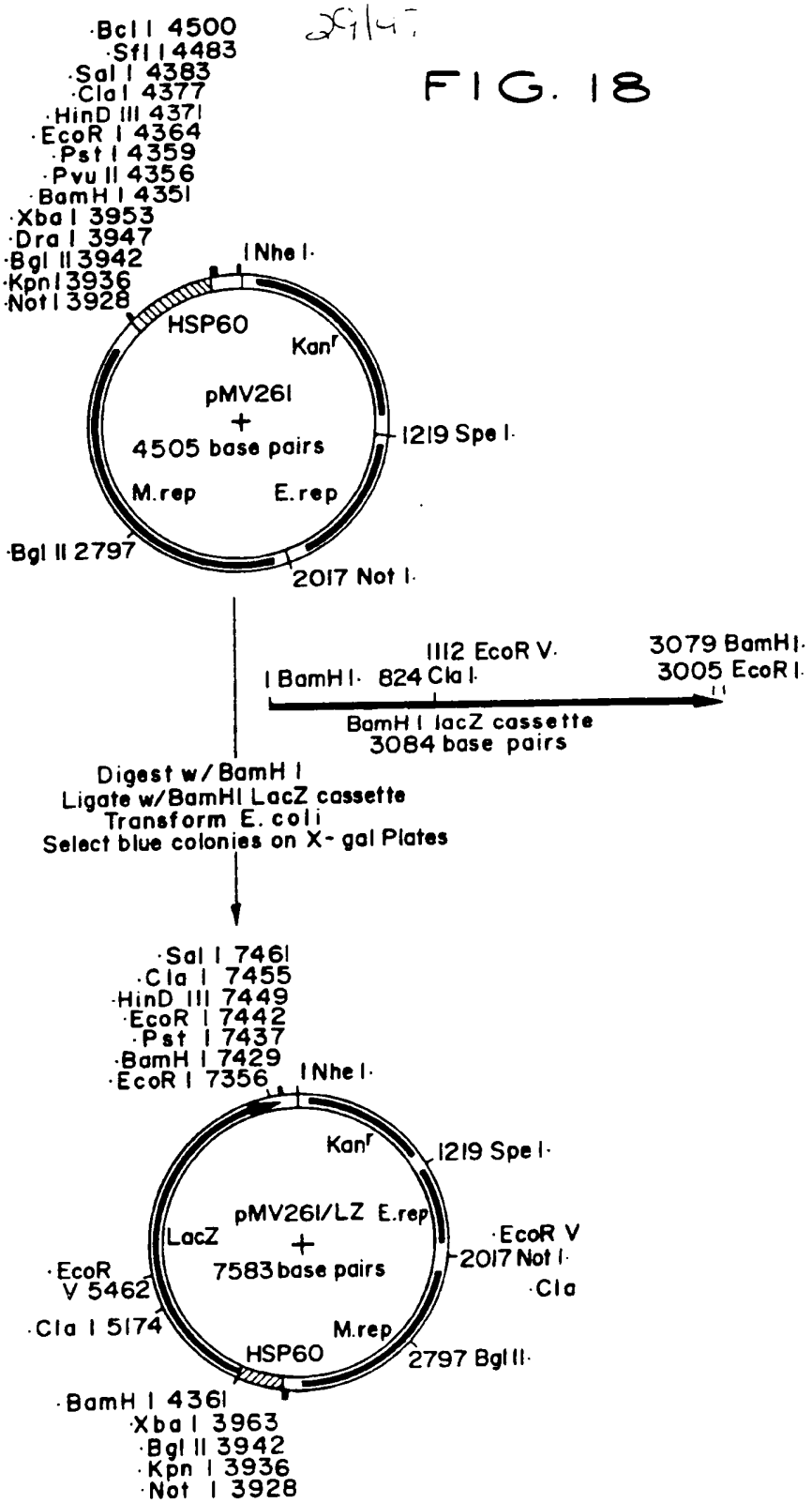

Bcl I 4500
Sfl I 4483
Sal I 4383
Cla I 4377
HinD III 4371
EcoR I 4364
Pst I 4359
Pvu II 4356
BamH I 4351
Xba I 3953
Dra I 3947
Bgl II 3942
Kpn I 3936
Not I 3928
I Nhe I
HSP60
Kan^r
pMV261
+
4505 base pairs
1219 Spe I
M.rep
E.rep
Bgl II 2797
2017 Not I
1112 EcoR V
3079 BamHI
I BamH I
824 Cla I
3005 EcoR I
BamH I lacZ cassette
3084 base pairs
Digest w/ BamH I
Ligate w/BamHI LacZ cassette
Transform E. coli
Select blue colonies on X- gal Plates
Sal I 7461
Cla I 7455
HinD III 7449
EcoR I 7442
Pst I 7437
BamH I 7429
EcoR I 7356
I Nhe I
Kan^r
1219 Spe I
pMV261/LZ E.rep
LacZ
+
7583 base pairs
EcoR V
2017 Not I
Cla
EcoR V 5462
Cla I 5174
M.rep
HSP60
2797 Bgl II
BamH I 4361
Xba I 3963
Bgl II 3942
Kpn I 3936
Not I 3928

FIG. 19

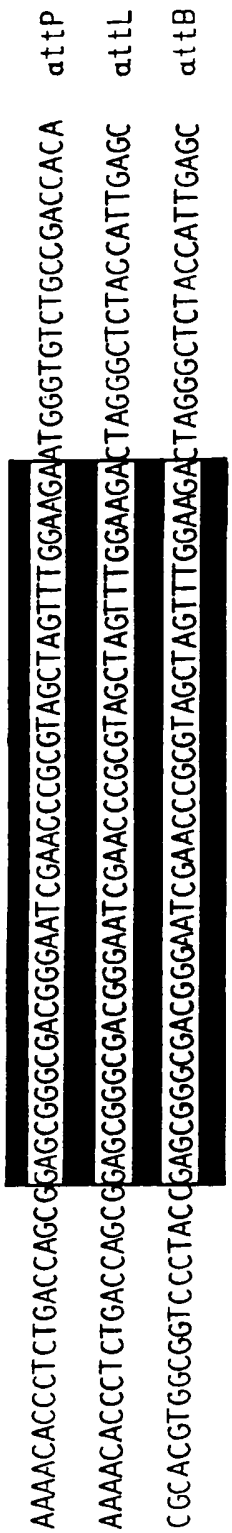
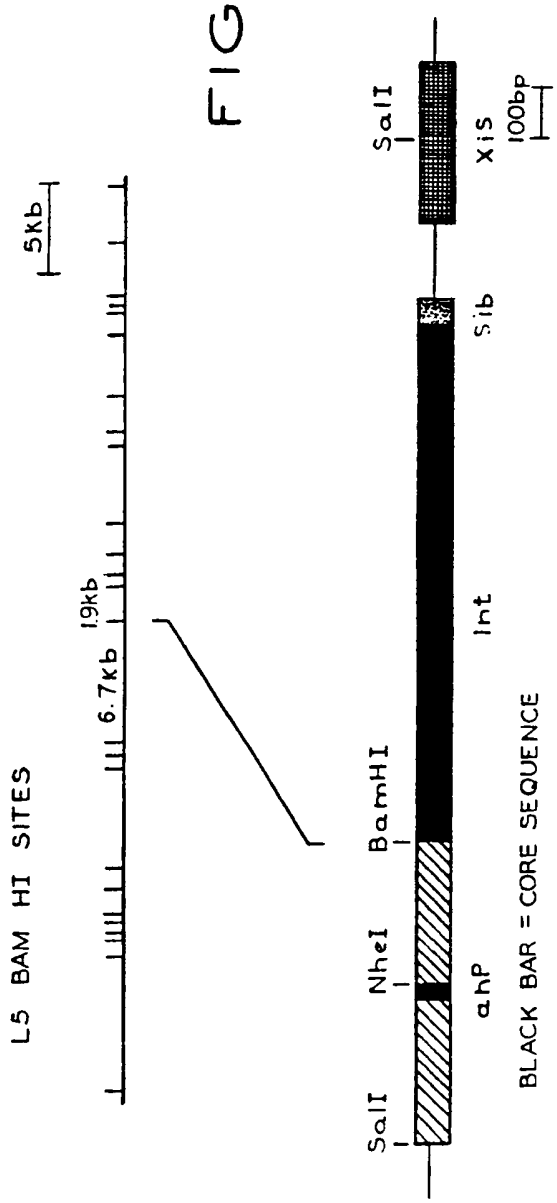

AAAACACCCTCTGACCAGCGGAGCGGGCGACGGGAATCGAACCCGCGTAGCTAGTTTGGAAGAATGGGTGTCTGCCGACCACA attP
AAAACACCCTCTGACCAGCGGAGCGGGCGACGGGAATCGAACCCGCGTAGCTAGTTTGGAAGACTAGGGCTCTACCATTGAGC attL
CGCACGTGGCGGTCCCTACCGAGCGGGCGACGGGAATCGAACCCGCGTAGCTAGTTTGGAAGACTAGGGCTCTACCATTGAGC attB

FIG. 21


5kb
L5 BAM HI SITES
1.9kb
6.7kb
SalI
NheI
BamHI
SalI
int
Sib
xis
100bp
ahP
BLACK BAR = CORE SEQUENCE

	DOMAIN 1	DOMAIN 2
INT (λ)	RLAMELAVVTGQRVGDLCEMKWSDIVDG	HELRSLSA-RLYEKQ-ISDKFAQHLLGHKS-DTMASQYR-
INT (HK22)	RLAMDLAVVTGQRVGDLCRMKWSDIVDG	HELRSLSA-RLYRNQ-IGDKFAQRLLGHKS-DSMAARYRD
INT (φ 80)	VFLVKFIMLTGCRTAEIRLSERSWFRLD	HDMRRTIATNLSELG-CPPHVIEKLLGHQM-VGVMAHYN-
INT (P2)	KKIAILCLSTGARWGEAARLKAENIIHN	HALRHSFATHFMING-GSIITLQRILGHTR-IEQTMVYAH
INT (P4)	MIAVKLSLLTFVRSSELRFARWDEFDFD	HGFRTMARGALGESGLWSDDAIERQSLHSERNNVRAAYIH
INT (P22)	KSVVEFALSTGLRRSNIINLEWQQIDMQ	HDLRHTWASWLVQAG-VPISVLQEMGGWES-IEMVRRYAH
INT (186)	ETVVRICLATGARWSEAESLRKSQLAKY	HVLRHTFASHFMMNG-GNILVLQRVLGHTD-IKMTMRYAH
INT (HP1)	GLIVRICLATGARWSEAETLTQSQVMPY	HVLRHTFASHFMMNG-GNILVLKEILGHST-IEMTMRYAH
INT (L54a)	AGAVEVQALTGMRIGELLALQVKDVDLK	HTLRHTHISLLAEMN-ISLKAIMKRVGHRDEKTTIKVYTH
Cre (P1)	TAGVEKALSLGVTKLVERWISVSGVADD	HSARVGAARDMARAG-VSIPEIMQAGGWTN-VNIVMNYIR
D Prot. (F)	KMLLATLWNTGARINEALALTRGDFSLA	HTFRHSYAMHMLYAG-IPLKVLQSLMGHKS-ISSTEVYTK
Fim B	YCLTLLCFIHGFRASEICRLRISDIDLK	HMLRHSCGFALANMG-IDTRLIQDYLGHRN-IRHTVRYTA
Fim E	YCLILLAYRHGMRISELLDLHYQDLDLN	HMLRHACGYELAERG-ADTRLIQDYLGHRN-IRHTVRYTA
Tn2603 ORF3	RLFAQLLYGTCMRISEGLQLRVKDLDFD	HTLRHSFATALLRSG-YDIRTVQDLLGHSD-VSTTMIYTH
Tn554 TnpA	KLILMLMYEGGLRIGEVLSLRLEDIVTW	HMLRHTHATQLIREG-WDVAFVQKRLGHAHVQTTLNTYVH
Tn554 TnpB	ATMTMIVQECGMRISELCTLKKGCLLED	HAFRHTVGTRMINNG-MPQHIVQKFLGHES-PEMTSRYAH
Tn4430 TnpI	YAIATLLAYTGVRISEALSIKMNDFNLQ	HQLRHFFCTNAIEKG-FSIHEVANQAGHSN-IHTTLLYT-
Rci	YVIFHLALETAMRQQEILALRWEHIDLR	HDLRHEAISRFFELGSLNVMEIAAISGHRS-MNMLKRYTH
Tn1545 ORF2	YDEILILLKTGLRISEFGGLTLPDLDFE	HIGRHLMTSFLSMKGLTELTNVVGNWSDKRASAVATTYTH
Flp	----------	HIGRHLMTSFLSMKGLTELTNVVGNWSDKRASAVATTYTH
CONSENSUS	--Iv-L-I-TGmR-SEI--Lr--di---	H-LRHt-At-L---G---i--IQ-ILgh---i--T--Y-H
L5	RIAAYILAWTSLRFGELIELRRKDIVDD	HDLRAVGATFAAQAG-ATTKELMARLGHTT-PRMAMKYQM

FIG. 20

32147

FIG. 22B

MATCH WITH FIG. 22A

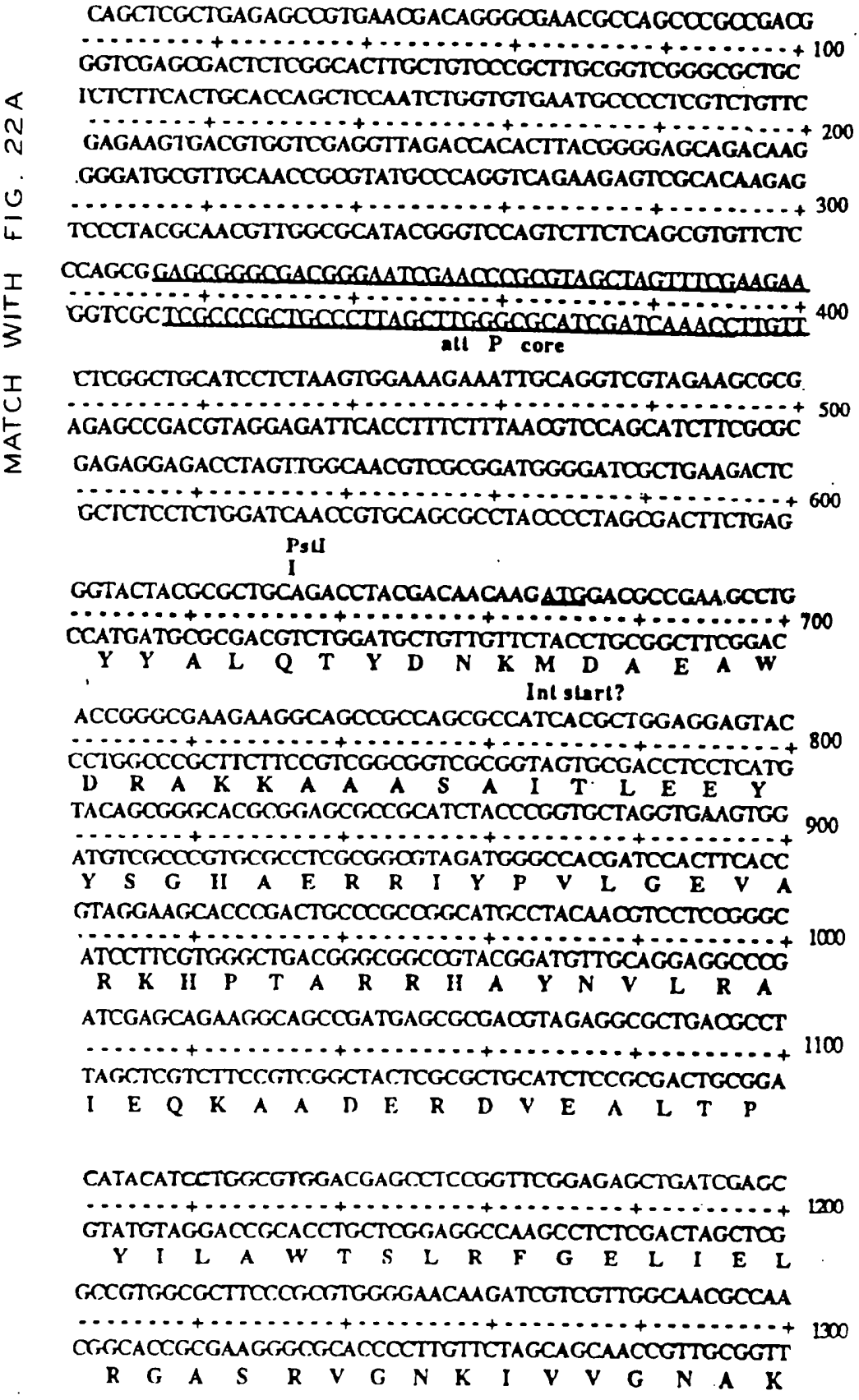
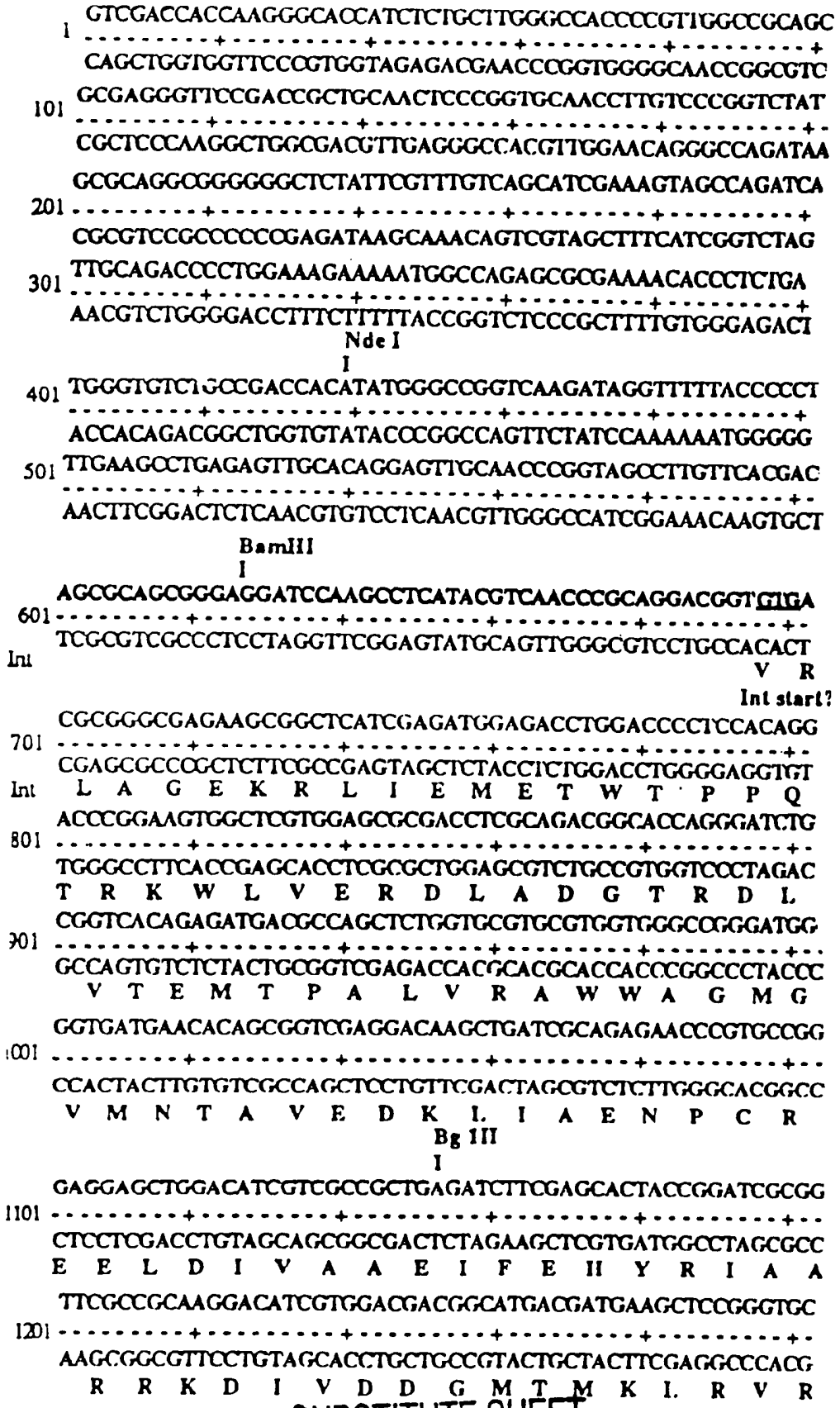


FIG. 22A



MATCH WITH FIG. 22B

FIG. 23

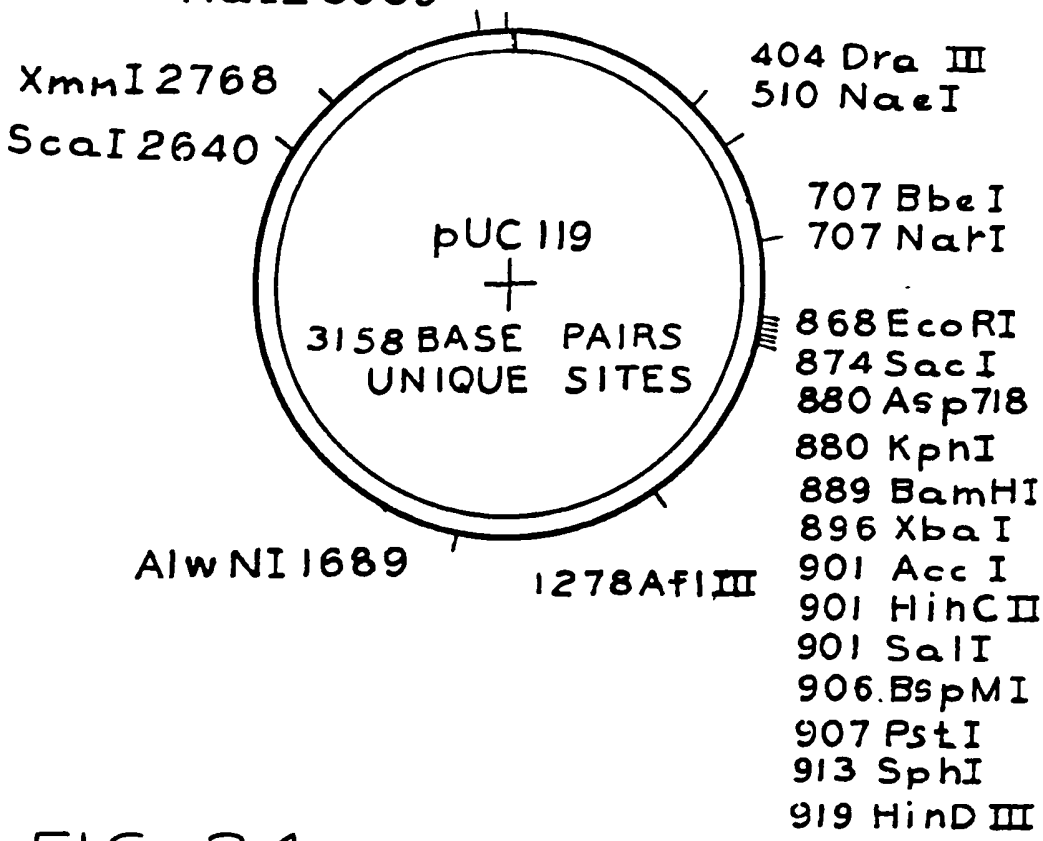

Eco0109I 3148
AatII 3089
XmnI 2768
ScaI 2640
404 Dra III
510 NaeI
707 BbeI
707 NarI
pUC 119
+
3158 BASE PAIRS
UNIQUE SITES
868 EcoRI
874 SacI
880 Asp718
880 KpnI
889 BamHI
896 XbaI
901 Acc I
901 HinCII
901 SalI
906 BspMI
907 PstI
913 SphI
919 HinD III
AlwNI 1689
1278 AflIII

FIG. 24

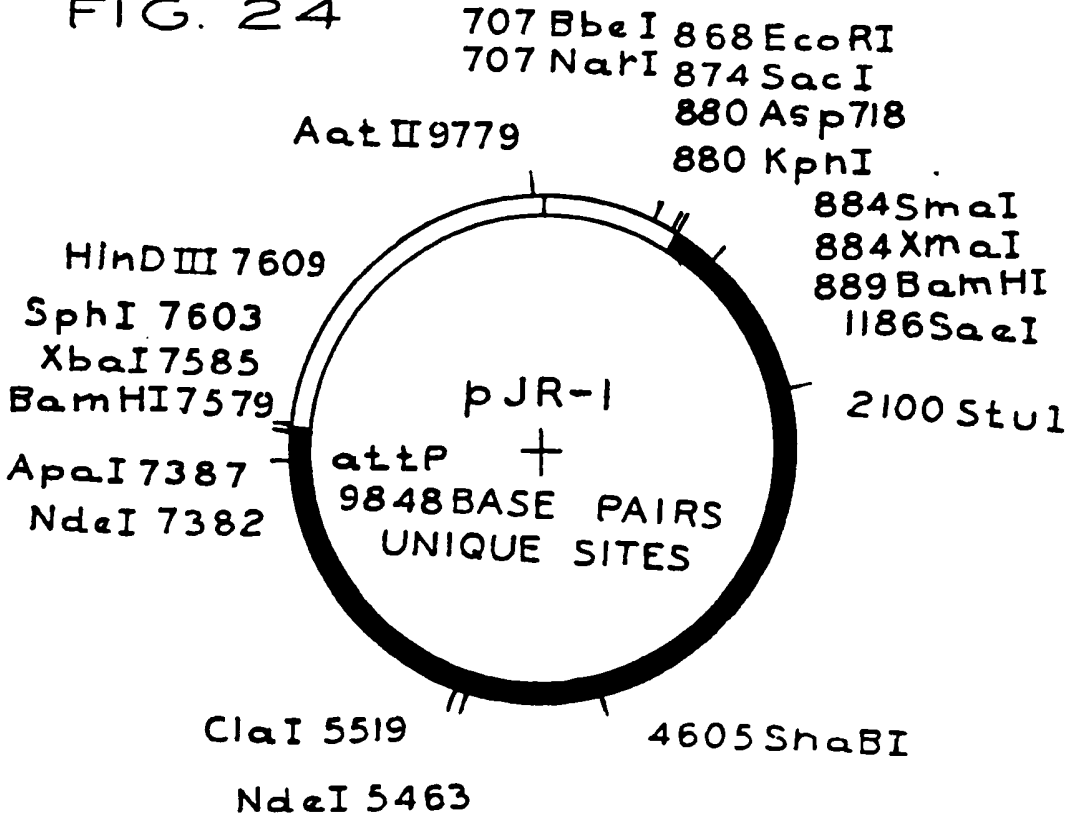

707 BbeI
707 NarI
868 EcoRI
874 SacI
880 Asp718
880 KpnI
884 SmaI
884 XmaI
889 BamHI
1186 SacI
AatII 9779
HinD III 7609
SphI 7603
XbaI 7585
BamHI 7579
ApaI 7387
NdeI 7382
pJR-1
+
attP
9848 BASE PAIRS
UNIQUE SITES
2100 StuI
ClaI 5519
NdeI 5463
4605 ShaBI

FIG. 25

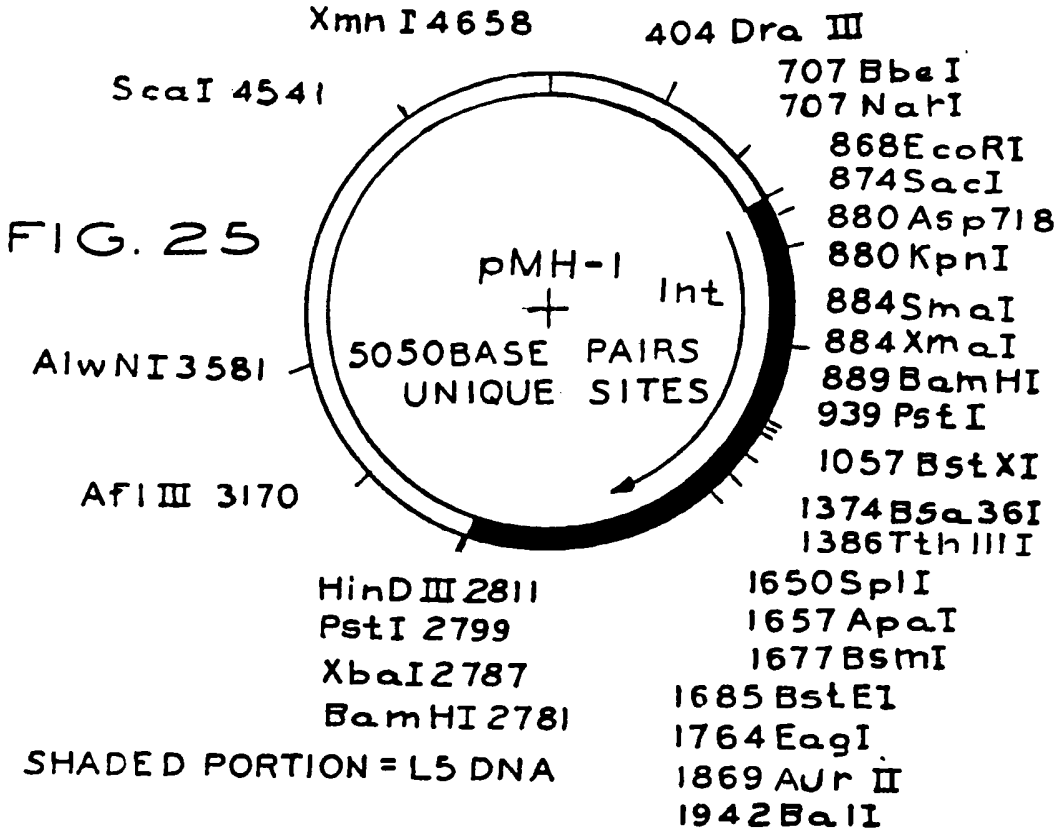

Xmn I 4658
404 Dra III
707 Bbe I
707 Nar I
868 EcoRI
874 SacI
880 Asp718
880 KpnI
884 SmaI
884 XmaI
889 BamHI
939 Pst I
1057 Bst XI
1374 Bsa 36I
1386 Tth 111 I
1650 Spl I
1657 ApaI
1677 BsmI
1685 BstE1
1764 EagI
1869 AUr II
1942 Ba1 I
ScaI 4541
AlwNI 3581
Af1 III 3170
HinD III 2811
Pst I 2799
Xba I 2787
BamHI 2781
pMH-1
+
5050 BASE PAIRS
UNIQUE SITES
Int
SHADED PORTION = L5 DNA

FIG. 27

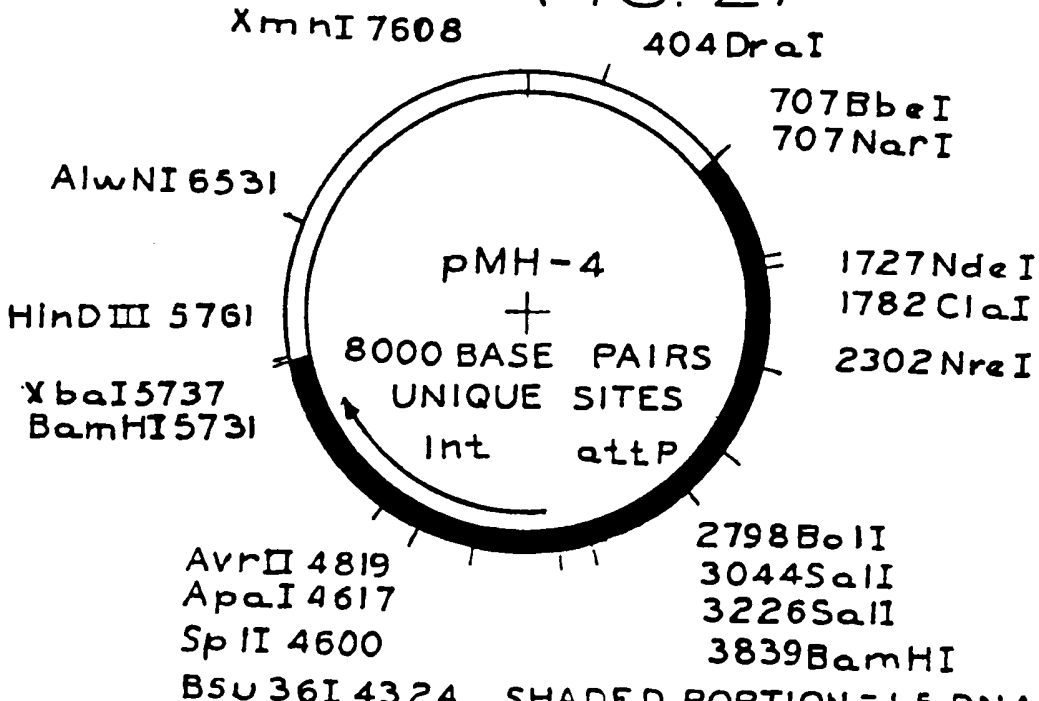

Xmn I 7608
404 Dra I
707 Bbe I
707 Nar I
1727 Nde I
1782 Cla I
2302 Nre I
2798 Bo1 I
3044 Sal I
3226 Sal I
3839 BamHI
AlwNI 6531
HinD III 5761
Xba I 5737
BamHI 5731
Avr II 4819
Apa I 4617
Sp II 4600
Bsu 36I 4324
pMH-4
+
8000 BASE PAIRS
UNIQUE SITES
Int
attP
SHADED PORTION = L5 DNA

FIG. 28

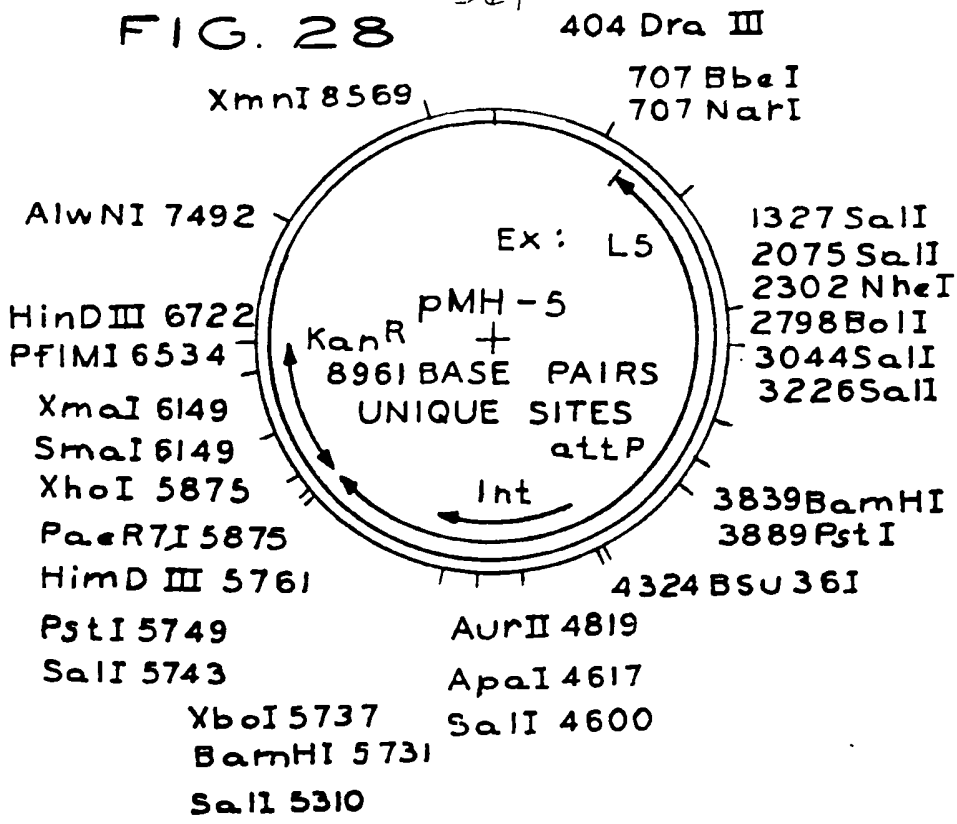
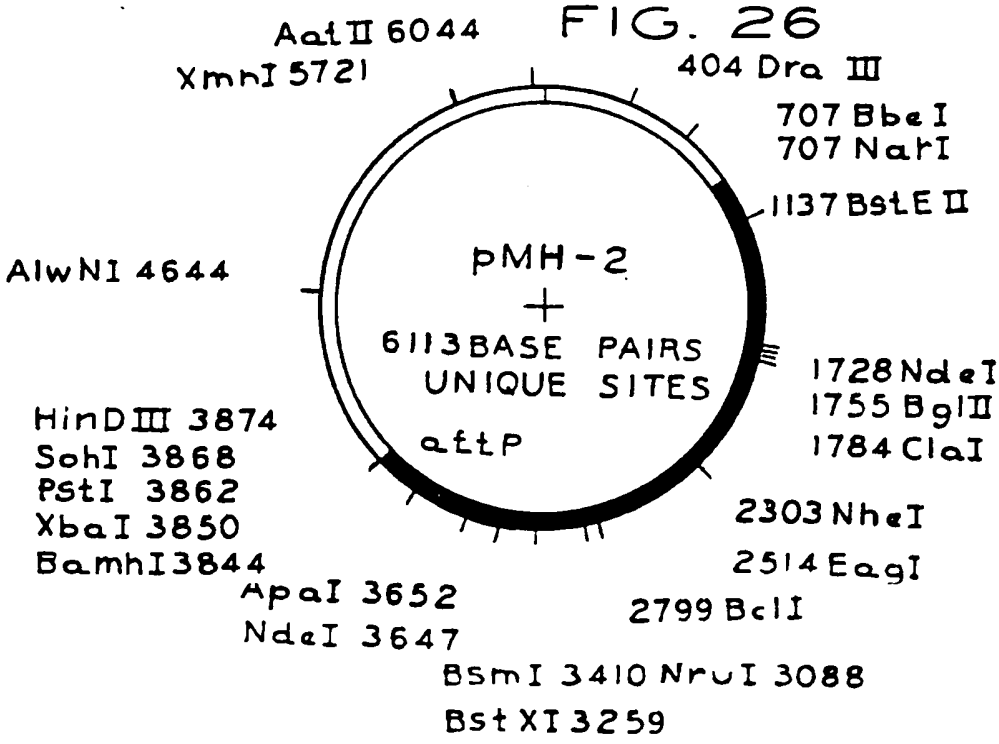


FIG. 26



SHADED PORTION = L5 DNA

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FIG. 29

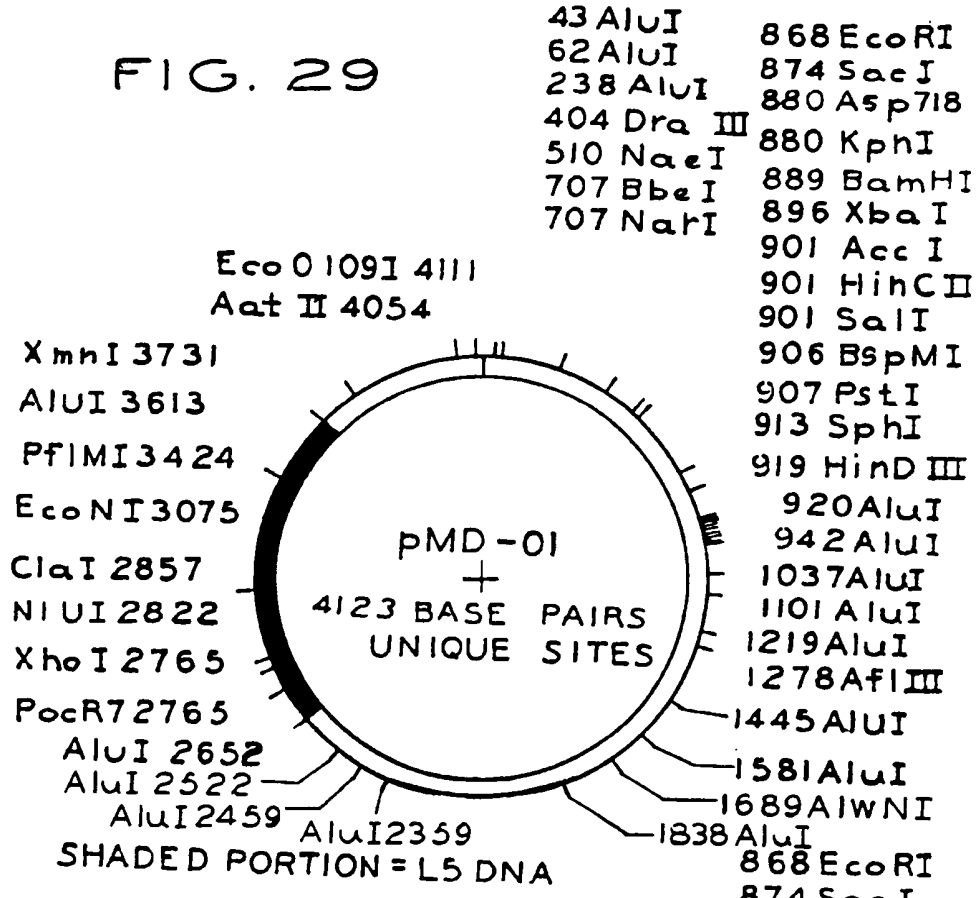

FIG. 31

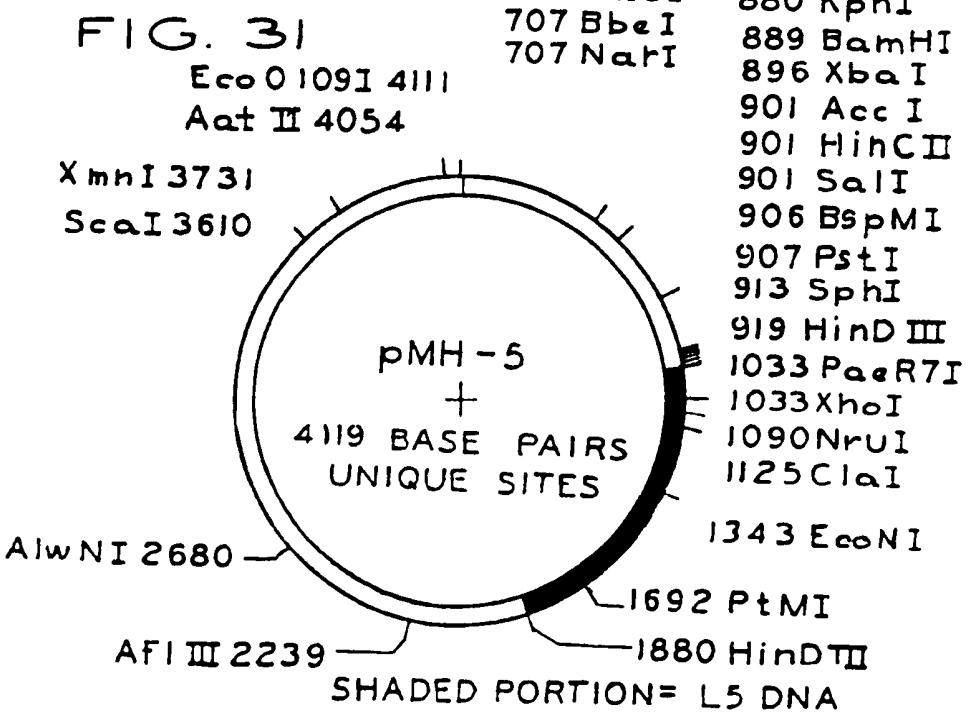

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FIG. 30

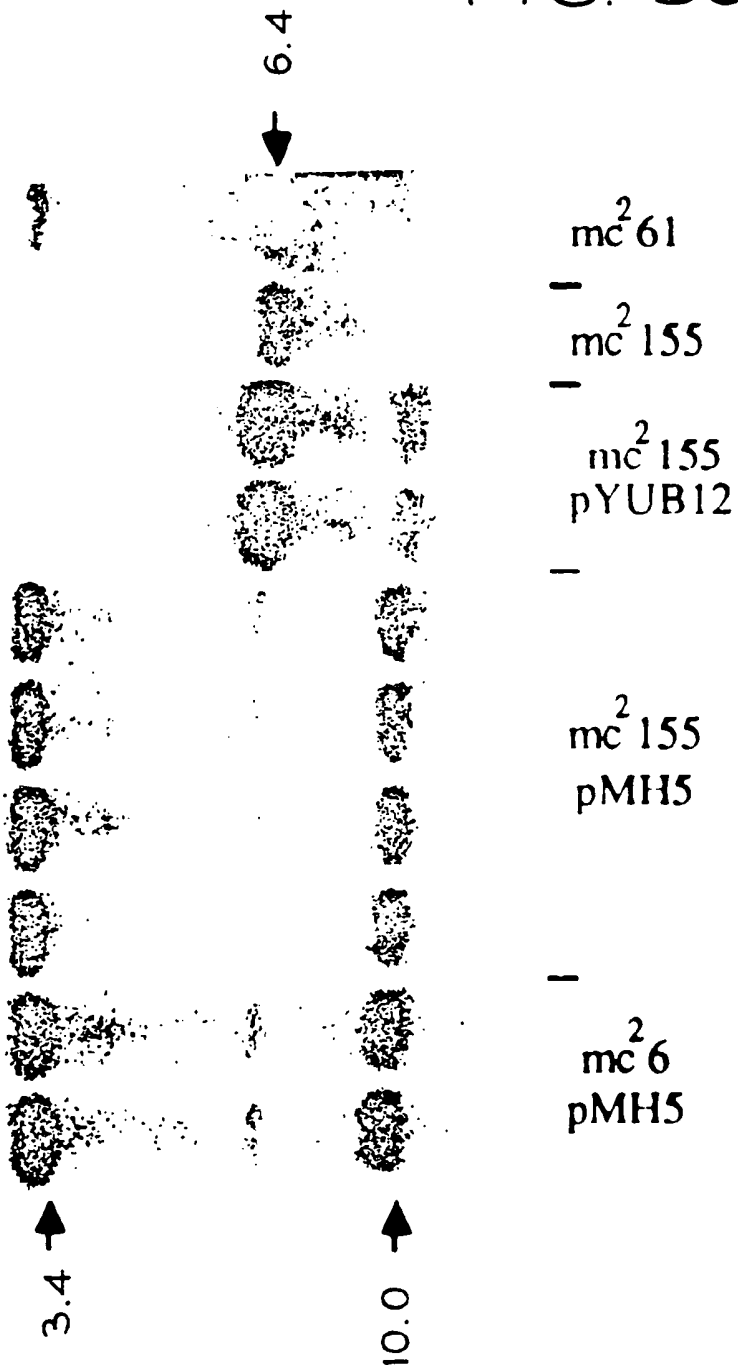


FIG. 32

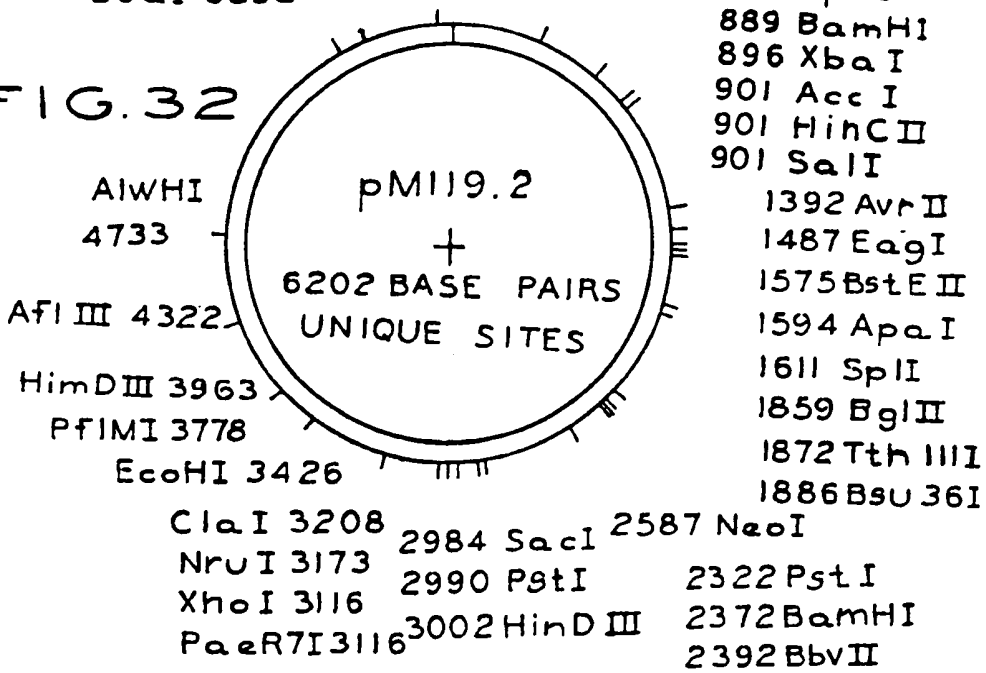
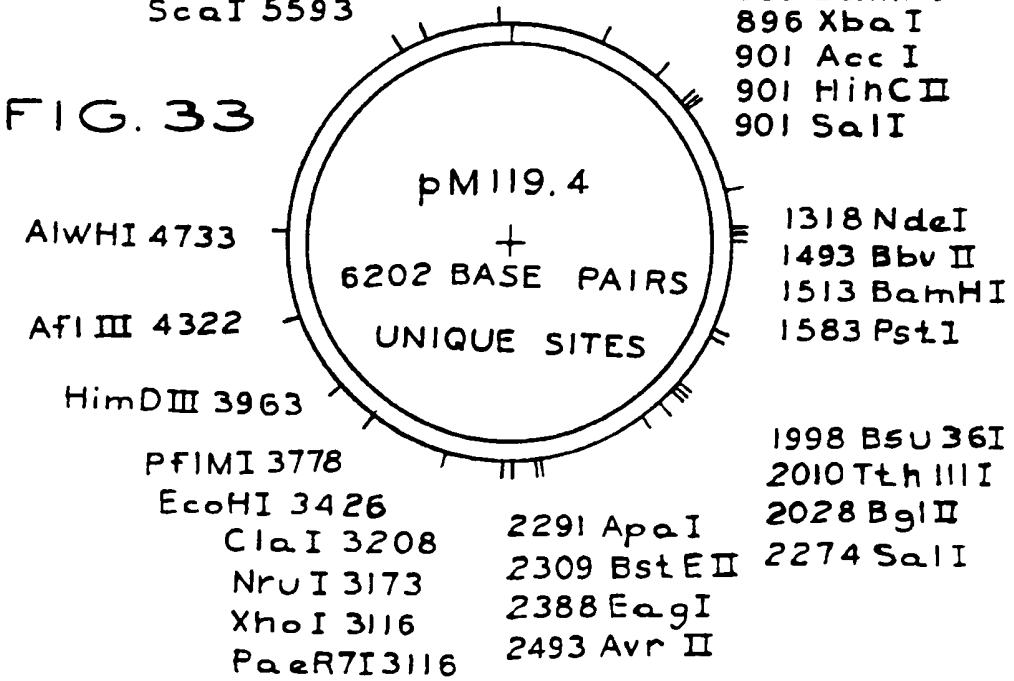

Aat II 6133
XmnI 5810
ScaI 5593
AlwHI 4733
AfI III 4322
HimDIII 3963
PfIMI 3778
EcoHI 3426
ClaI 3208
NruI 3173
XhoI 3116
PaeR7I 3116
2984 SacI
2990 PstI
3002 HinD III
2587 NaeI
2322 PstI
2372 BamHI
2392 BbvII
404 DraI
707 BbeI
707 NarI
868 EcoRI
874 SacI
880 Asp718
880 KpnI
889 BamHI
896 XbaI
901 AccI
901 HinCII
901 SalI
1392 AvrII
1487 EagI
1575 BstEII
1594 ApaI
1611 SpII
1859 BglII
1872 TthIIII
1886 BsU36I
pM119.2
+
6202 BASE PAIRS
UNIQUE SITES

FIG. 33


Aat II 6133
XmnI 5810
ScaI 5593
AlwHI 4733
AfI III 4322
HimDIII 3963
PfIMI 3778
EcoHI 3426
ClaI 3208
NruI 3173
XhoI 3116
PaeR7I 3116
2291 ApaI
2309 BstEII
2388 EagI
2493 AvrII
404 DraI
707 BbeI
707 NarI
868 EcoRI
874 SacI
880 Asp718
880 KpnI
889 BamHI
896 XbaI
901 AccI
901 HinCII
901 SalI
1318 NdeI
1493 BbvII
1513 BamHI
1583 PstI
1998 BsU36I
2010 TthIIII
2028 BglII
2274 SalI
pM119.4
+
6202 BASE PAIRS
UNIQUE SITES

40\47

FIG. 34

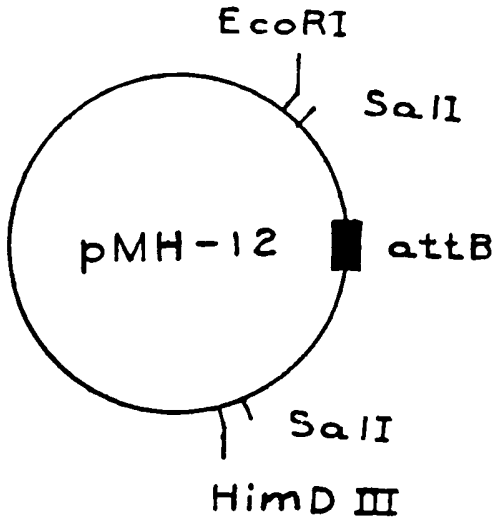


FIG. 36

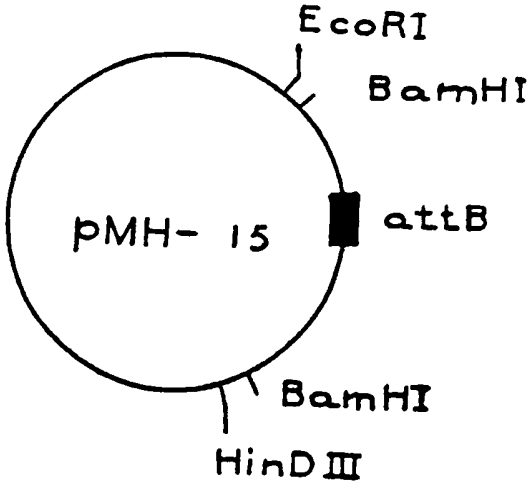


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FIG. 37

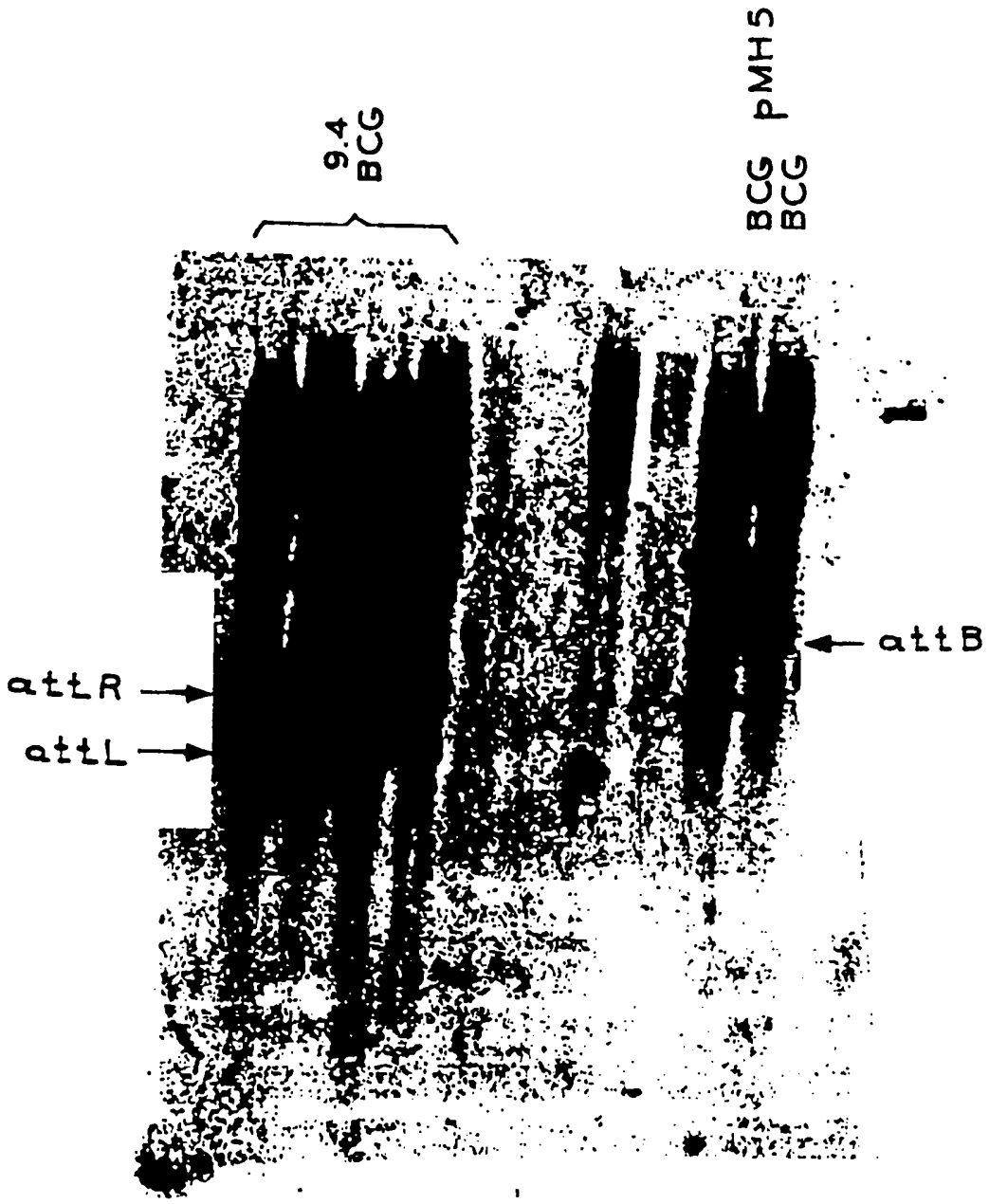
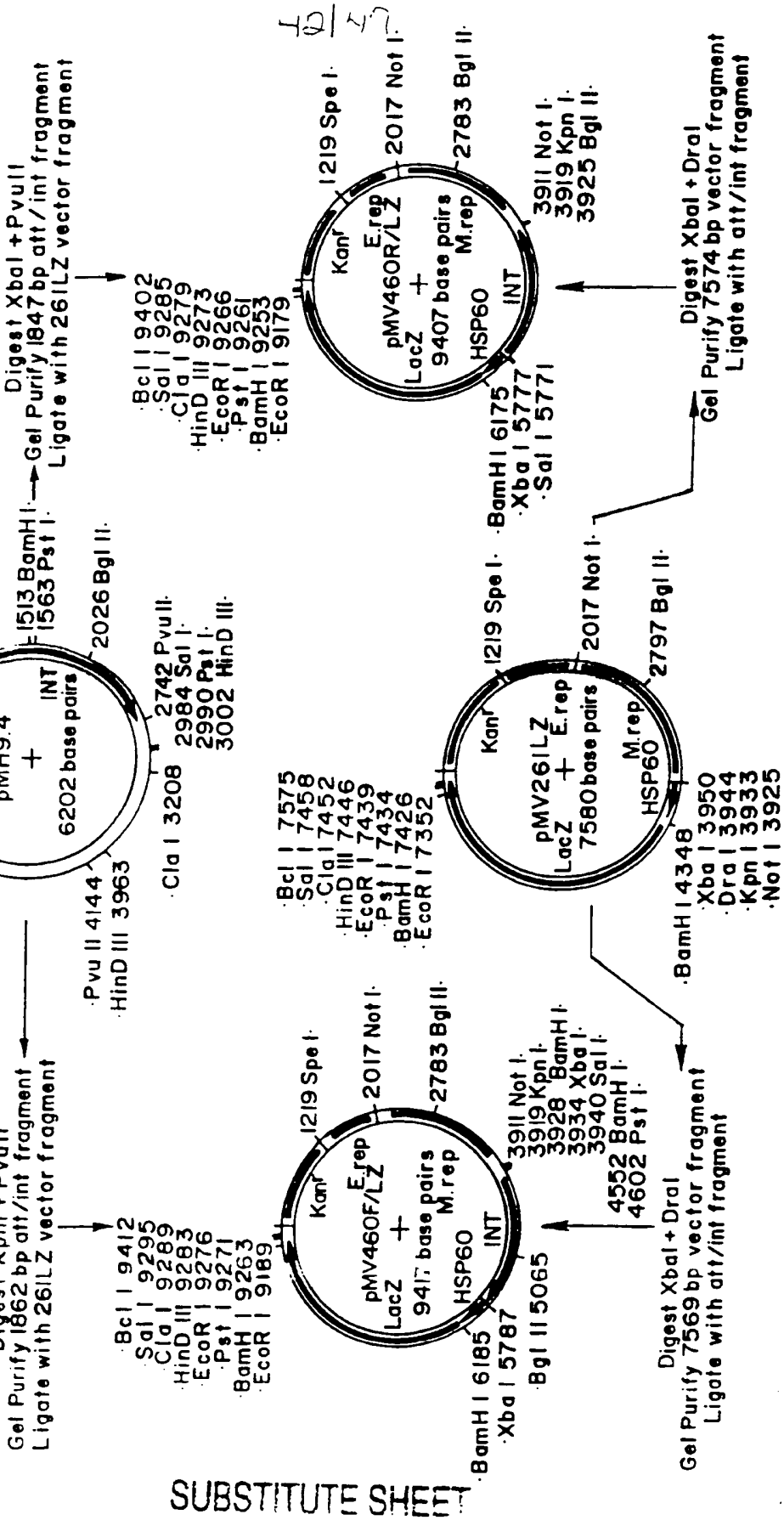


FIG. 38


pMH9.4
+
6202 base pairs
INT
778 PvuII
868 EcoRI
880 KpnI
889 BamHI
895 XbaI
901 SalI
1513 BamHI
1563 PstI
2026 BglII
2742 PvuII
2984 SalI
2990 PstI
3002 HindIII
Pvu II 4144
HindIII 3963
Cla I 3208
Digest XbaI + PvuII
Gel Purify 1847 bp att/int fragment
Ligate with 261LZ vector fragment
BclI 9402
SalI 9285
ClaI 9279
HindIII 9273
EcoRI 9266
PstI 9261
BamHI 9253
EcoRI 9179
pMV460R/LZ
LacZ
+
9407 base pairs
Kan
E.rep
M.rep
HSP60
INT
1219 SpeI
2017 NotI
2783 BglII
3911 NotI
3919 KpnI
3925 BglII
BamHI 6175
XbaI 5777
SalI 5771
Digest XbaI + DraI
Gel Purify 7574 bp vector fragment
Ligate with att/int fragment
pMV261LZ
LacZ
+
7580 base pairs
Kan
E.rep
M.rep
HSP60
1219 SpeI
2017 NotI
2797 BglII
BclI 7575
SalI 7458
ClaI 7452
HindIII 7446
EcoRI 7439
PstI 7434
BamHI 7426
EcoRI 7352
BamHI 4348
XbaI 3950
DraI 3944
KpnI 3933
NotI 3925
Digest KpnI + PvuII
Gel Purify 1862 bp att/int fragment
Ligate with 261LZ vector fragment
BclI 9412
SalI 9295
ClaI 9289
HindIII 9283
EcoRI 9276
PstI 9271
BamHI 9263
EcoRI 9189
pMV460F/LZ
LacZ
+
9417 base pairs
Kan
E.rep
M.rep
HSP60
INT
1219 SpeI
2017 NotI
2783 BglII
3911 NotI
3919 KpnI
3928 BamHI
3934 XbaI
3940 SalI
4552 BamHI
4602 PstI
BamHI 6185
XbaI 5787
BglII 5065
Digest XbaI + DraI
Gel Purify 7569 bp vector fragment
Ligate with att/int fragment

43147

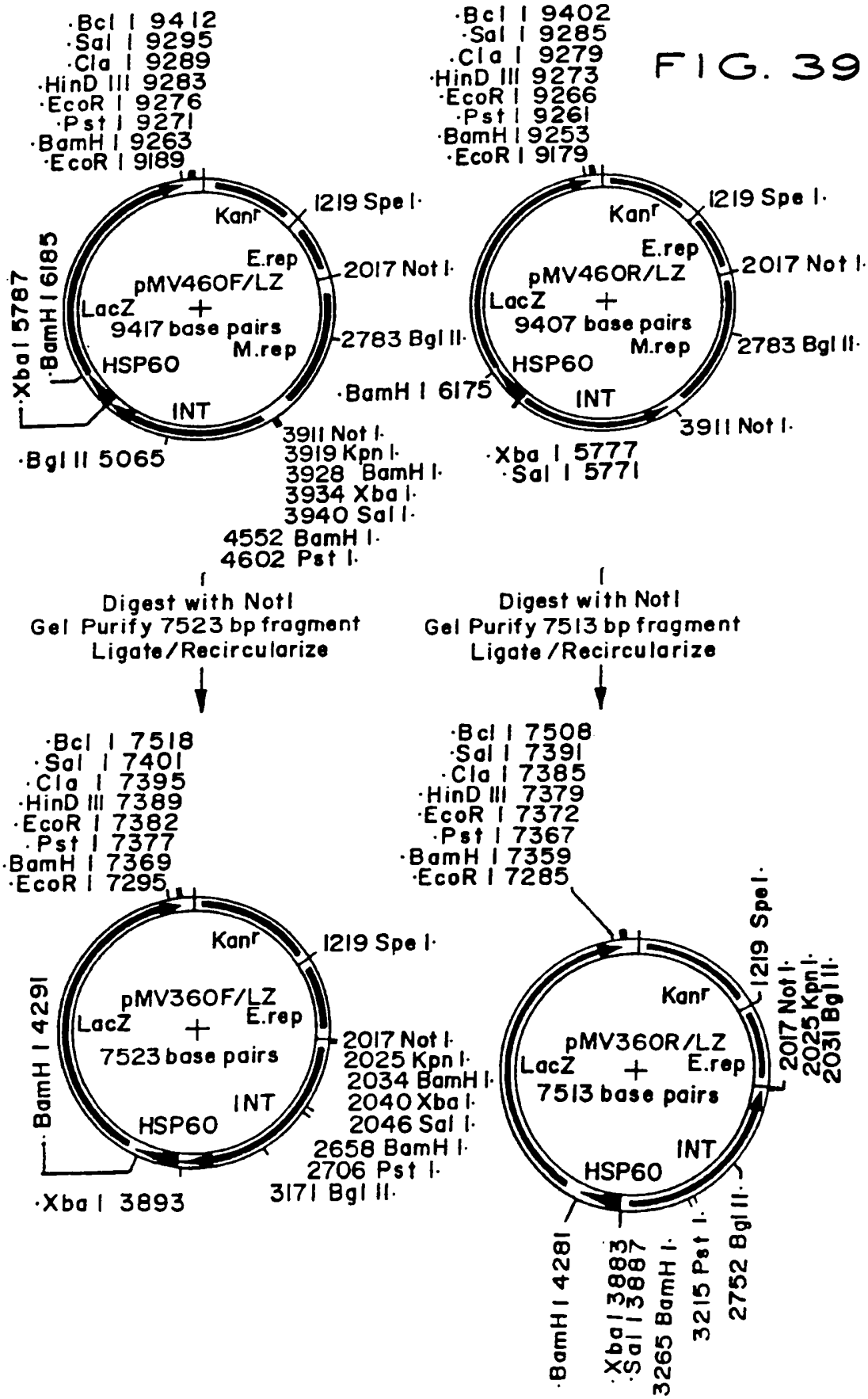

·Bcl I 9412
·Sal I 9295
·Cla I 9289
·HinD III 9283
·EcoR I 9276
·Pst I 9271
·BamH I 9263
·EcoR I 9189
·Bcl I 9402
·Sal I 9285
·Cla I 9279
·HinD III 9273
·EcoR I 9266
·Pst I 9261
·BamH I 9253
·EcoR I 9179
FIG. 39
Kanr
E.rep
pMV460F/LZ
LacZ +
9417 base pairs
M.rep
HSP60
INT
1219 Spe I·
2017 Not I·
2783 Bgl II·
3911 Not I·
3919 Kpn I·
3928 BamH I·
3934 Xba I·
3940 Sal I·
4552 BamH I·
4602 Pst I·
·Xba I 5787
·BamH I 6185
·Bgl II 5065
pMV460R/LZ
9407 base pairs
·BamH I 6175
·Xba I 5777
·Sal I 5771
Digest with NotI
Gel Purify 7523 bp fragment
Ligate/Recircularize
Digest with NotI
Gel Purify 7513 bp fragment
Ligate/Recircularize
·Bcl I 7518
·Sal I 7401
·Cla I 7395
·HinD III 7389
·EcoR I 7382
·Pst I 7377
·BamH I 7369
·EcoR I 7295
pMV360F/LZ
7523 base pairs
2017 Not I·
2025 Kpn I·
2034 BamH I·
2040 Xba I·
2046 Sal I·
2658 BamH I·
2706 Pst I·
3171 Bgl II·
·BamH I 4291
·Xba I 3893
·Bcl I 7508
·Sal I 7391
·Cla I 7385
·HinD III 7379
·EcoR I 7372
·Pst I 7367
·BamH I 7359
·EcoR I 7285
pMV360R/LZ
7513 base pairs
1219 Spe I·
2017 Not I·
2025 Kpn I·
2031 Bgl II·
·BamH I 4281
·Xba I 3883
·Sal I 3887
3265 BamH I·
3215 Pst I·
2752 Bgl II·

FIG. 40

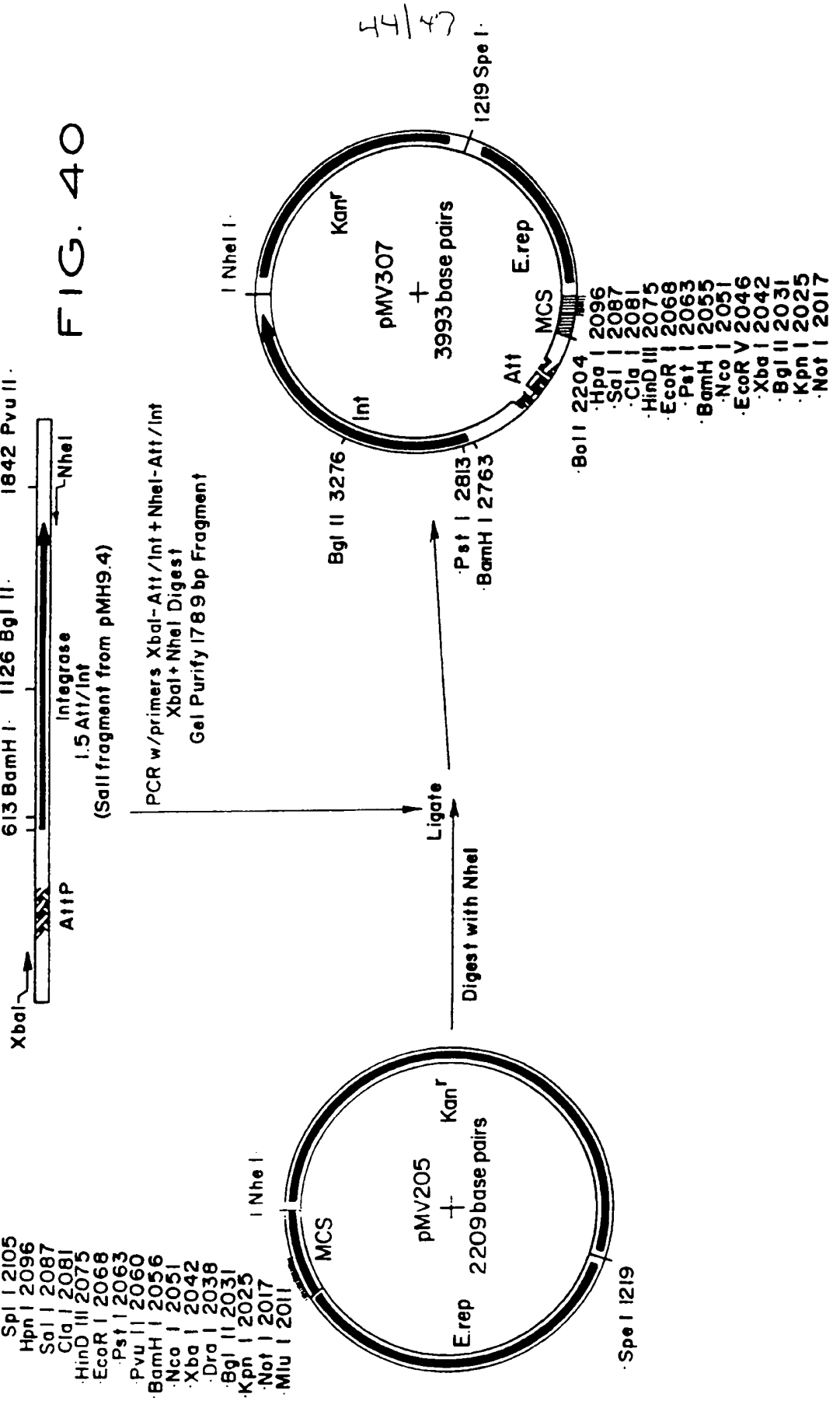

663 Pst I
613 BamH I
1126 Bgl II
1842 Pvu II
XbaI
AttP
Integrase
1.5 Att/Int
(SalI fragment from pMH9.4)
NheI
PCR w/primers XbaI-Att/Int+NheI-Att/Int
XbaI+NheI Digest
Gel Purify 1789 bp Fragment
Ligate
Digest with NheI
I Nhe I
Kanr
pMV307
+
3993 base pairs
E.rep
MCS
Att
Int
Bgl II 3276
Pst I 2813
BamH I 2763
1219 SpeI
BclI 2204
Hpa I 2096
Sal I 2087
Cla I 2081
HinD III 2075
EcoR I 2068
Pst I 2063
BamH I 2055
Nco I 2051
EcoR V 2046
Xba I 2042
Bgl II 2031
Kpn I 2025
Not I 2017
BclI 2204
Spl I 2105
Hpa I 2096
Sal I 2087
Cla I 2081
HinD III 2075
EcoR I 2068
Pst I 2063
Pvu II 2060
BamH I 2056
Nco I 2051
Xba I 2042
Dra I 2038
Bgl II 2031
Kpn I 2025
Not I 2017
Mlu I 2011
I Nhe I
MCS
E.rep
pMV205
+
2209 base pairs
Kanr
SpeI 1219

FIG. 44

45147

DAY 10 LYMPH NODES

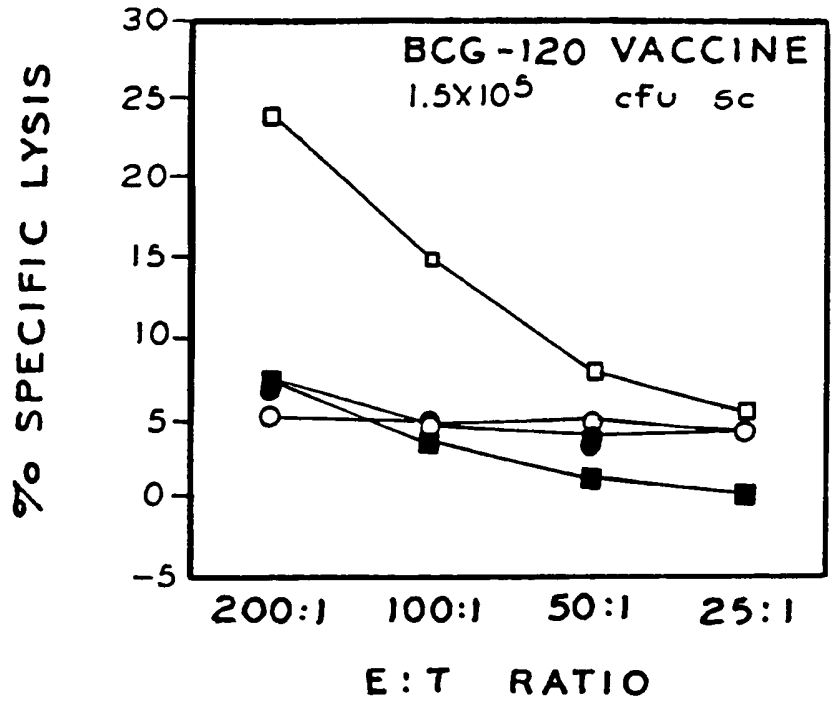


FIG. 41

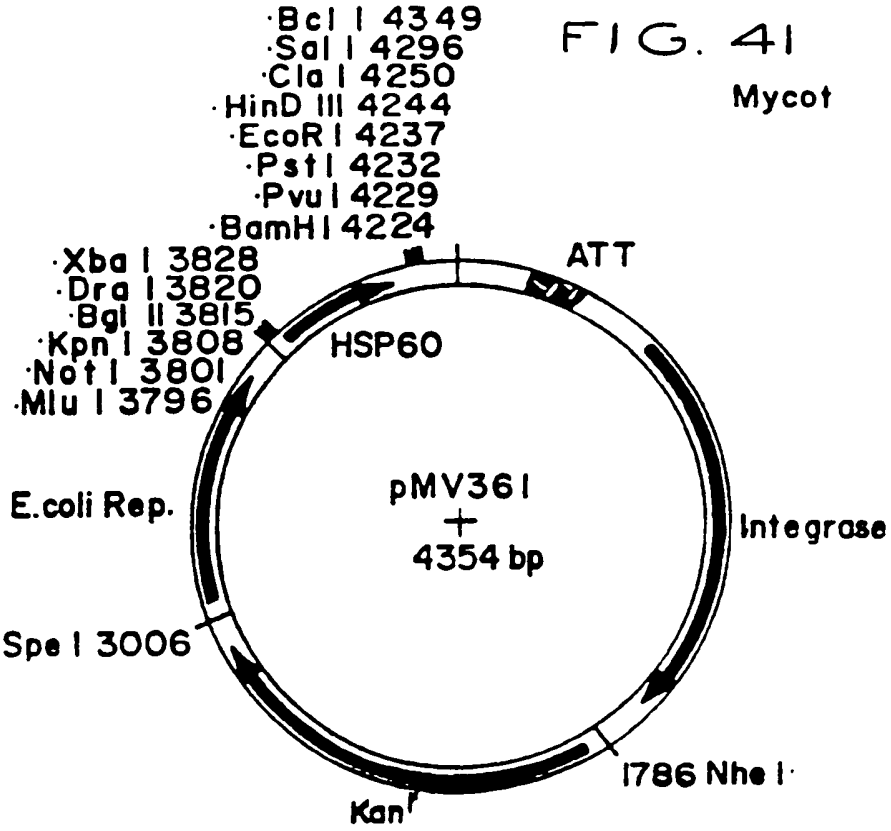


FIG. 35

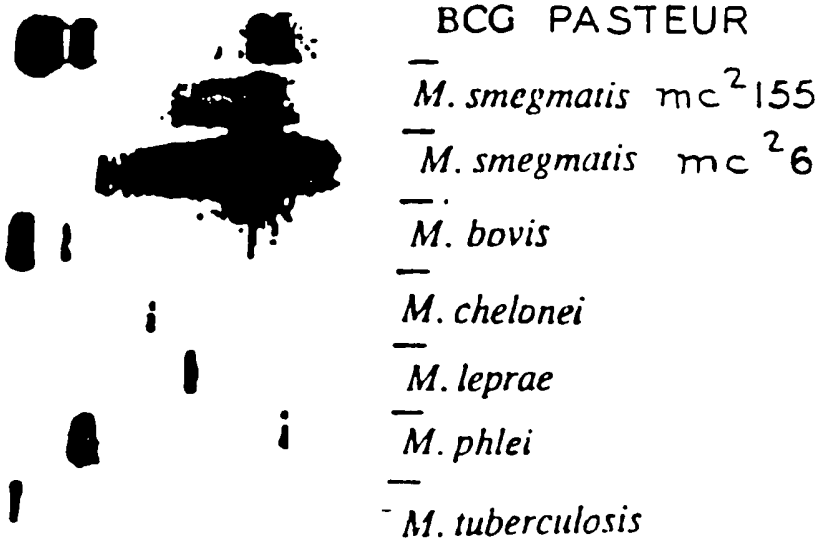
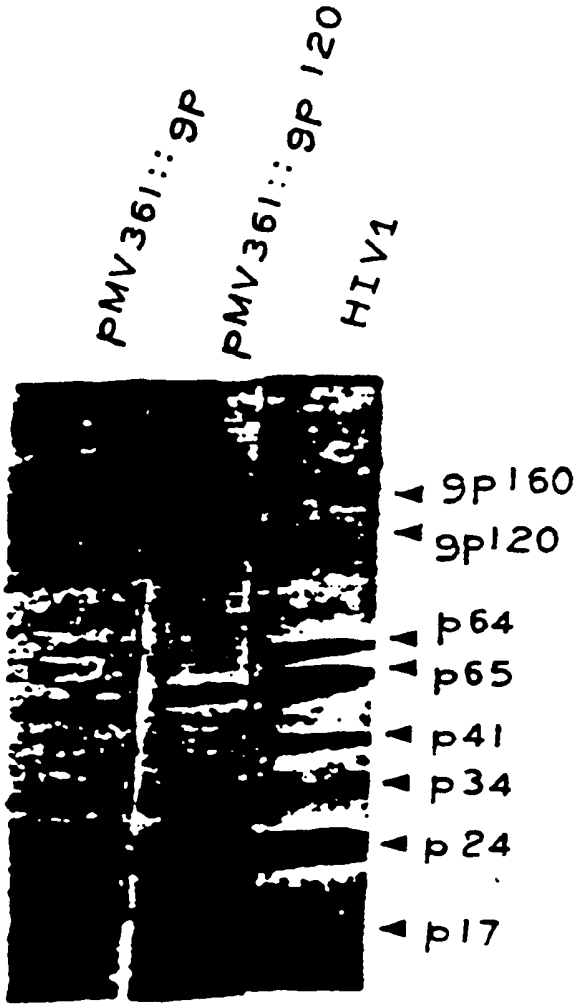
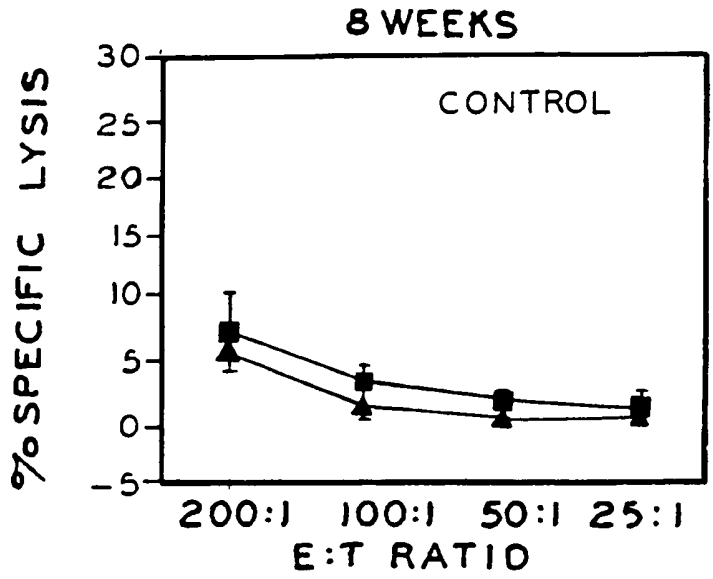
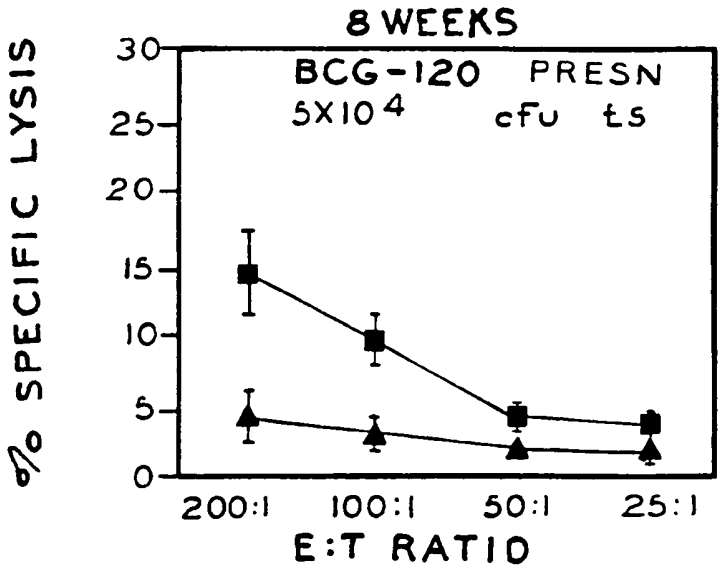

BCG PASTEUR
M. smegmatis mc²155
M. smegmatis mc²6
M. bovis
M. chelonei
M. leprae
M. phlei
M. tuberculosis

FIG. 42


PMV361::gp
PMV361::gp120
HIV1
gp160
gp120
p64
p65
p41
p34
p24
p17


8 WEEKS
CONTROL
% SPECIFIC LYSIS
30
25
20
15
10
5
0
-5
200:1 100:1 50:1 25:1
E:T RATIO


8 WEEKS
BCG-120 PRESN
5X10 4 cfu ts
% SPECIFIC LYSIS
30
25
20
15
10
5
0
200:1 100:1 50:1 25:1
E:T RATIO

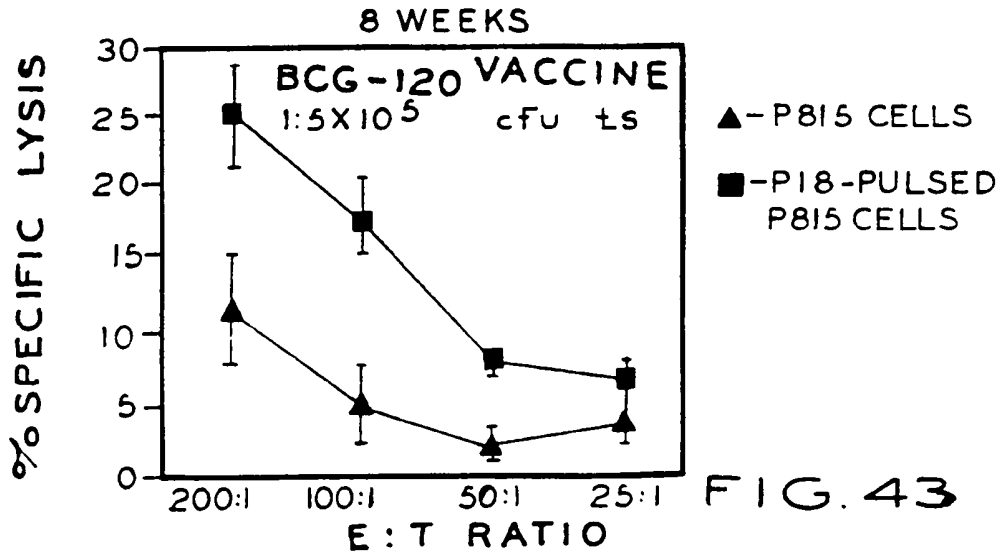

8 WEEKS
BCG-120 VACCINE
1:5X10 5 cfu ts
% SPECIFIC LYSIS
30
25
20
15
10
5
0
200:1 100:1 50:1 25:1
E:T RATIO
▲-P815 CELLS
■-P18-PULSED P815 CELLS

FIG. 43

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/04538

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/12
US CL :424/89; 435/863

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/89; 435/863

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS; Dialog (files = 155,154,5,399) (search terms: mycobacteria, vectors, HIV, recombinant)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 90/00594 (Bloom, et al) 25 January 1990, pages 1-95, especially pages 6, 11, 79, 81 and 92.	1-8
Y	Nature, Volume 328, issued 23 July 1987, Walker et al, "HIV-Specific cytotoxic T Lymphocytes In Seropositive Individuals", pages 345-348, see entire document.	1-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

06 August 1992

Date of mailing of the international search report

13 AUG 1992

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Form PCT/ISA/210 (second sheet)(July 1992)*